

4625-021-55X CIP of
4625-016-55X CIP of
4625-004-55X

TITLE OF THE INVENTION

POLYNUCLEIC ACIDS ISOLATED FROM A PORCINE REPRODUCTIVE AND
RESPIRATORY SYNDROME VIRUS (PRRSV), PROTEINS ENCODED BY THE
POLYNUCLEIC ACIDS, VACCINES BASED ON THE PROTEINS AND/OR
5 POLYNUCLEIC ACIDS, A METHOD OF PROTECTING A PIG FROM A
PRRSV AND A METHOD OF DETECTING A PRRSV

This is a continuation-in-part of application Serial
No. 08/131,625, filed on October 5, 1993, pending, which is
a continuation-in-part of application Serial No.
10 07/969,071, filed on October 30, 1992, now abandoned. The
entire contents of application Serial No. 08/131,625, filed
on October 5, 1993, are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

15 The present invention concerns DNA isolated from a
porcine reproductive and respiratory virus (PRRSV), a
protein and/or a polypeptide encoded by the DNA, a vaccine
which protects pigs from a PRRSV based on the protein or
DNA, a method of protecting a pig from a PRRSV using the
20 vaccine, a method of producing the vaccine, a method of
treating a pig infected by or exposed to a PRRSV, and a
method of detecting a PRRSV.

Discussion of the Background:

In recent years, North American and European swine herds have been susceptible to infection by new strains of reproductive and respiratory viruses (see A.A.S.P.,
5 September/October 1991, pp. 7-11; *The Veterinary Record*, February 1, 1992, pp. 87-89; *Ibid.*, November 30, 1991, pp. 495-496; *Ibid.*, October 26, 1991, p. 370; *Ibid.*, October 19, 1991, pp. 367-368; *Ibid.*, August 3, 1991, pp. 102-103; *Ibid.*, July 6, 1991; *Ibid.*, June 22, 1991, p. 578; *Ibid.*,
10 June 15, 1991, p. 574; *Ibid.*, June 8, 1991, p. 536; *Ibid.*, June 1, 1991, p. 511; *Ibid.*, March 2, 1991, p. 213). Among the first of the new strains to be identified was a virus associated with the so-called Mystery Swine Disease (MSD) or "blue-eared syndrome", now known as Swine Infertility
15 and Respiratory Syndrome (SIRS) or Porcine Reproductive and Respiratory Syndrome (PRRS).

An MSD consisting of reproductive failure in females and respiratory disease in nursing and weaned pigs appeared in the midwestern United States in 1987 (Hill et al., *Am. Assoc. Swine Practitioner Newsletter* 4:47 (1992); Hill et
20 al., *Proceedings Mystery Swine Disease Committee Meeting*, Denver, Colorado 29-31 (1990); Keffaber, *Am. Assoc. Swine Practitioner Newsletter* 1:1-9 (1989); Loula, *Agri-Practice* 12:23-34 (1991)). Reproductive failure was characterized
25 by abortions, stillborn and weak-born pigs. The respiratory disease in nursing and weaned pigs was

characterized by fever, labored breathing and pneumonia. A similar disease appeared in Europe in 1990 (Paton et al., Vet. Rec. 128:617 (1991); Wensvoort et al., *Veterinary Quarterly* 13:121-130 (1991); Blaha, *Proc. Am. Assoc. Swine Practitioners*, pp. 313-315 (1993)), and has now been
5 recognized worldwide.

This disease has also been called porcine epidemic abortion and respiratory syndrome (PEARS), blue abortion disease, blue ear disease (U.K.), abortus blau
10 (Netherlands), seuchenhafter spatabort der schweine (Germany), Heko-Heko disease, and in the U.S., Wabash syndrome, mystery pig disease (MPD), and swine plague (see the references cited above and Meredith, *Review of Porcine Reproductive and Respiratory Disease Syndrome*, Pig Disease
15 Information Centre, Department of Veterinary Medicine, Madingley Road, Cambridge CB3 0ES, U.K. (1992); Wensvoort et al., *Vet. Res.* 24:117-124 (1993); Paul et al., *J. Clin. Vet. Med.* 11:19-28 (1993)). In Europe, the corresponding virus has been termed "Lelystad virus."

20 At an international conference in May, 1992, researchers from around the world agreed to call this disease Porcine Reproductive and Respiratory Syndrome (PRRS). The disease originally appeared to be mainly a reproductive disease during its early phases, but has now
25 evolved primarily into a respiratory disease.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a relatively recently recognized swine pathogen associated with porcine reproductive and respiratory syndrome (PRRS). PRRSV is a significant pathogen in the swine industry. PRRSV infections are common in the U.S. swine herds. Outbreaks of PRRS in England have led to cancellation of pig shows.

The symptoms of PRRS include a reluctance to eat (anorexia), a mild fever (pyrexia), cyanosis of the extremities (notably bluish ears), stillbirths, abortion, high mortality in affected litters, weak-born piglets and premature farrowing. The majority of piglets born alive to affected sows die within 48 hours. PRRS clinical signs include mild influenza-like signs, rapid respiration ("thumping"), and a diffuse interstitial pneumonitis. PRRS virus has an incubation period of about 1-2 weeks from contact with a PRRSV-infected animal. The virus appears to be an enveloped RNA arterivirus (*The Veterinary Record*, February 1, 1992). The virus has been grown successfully in pig alveolar macrophages and CL2621 cells (Benfield et al, *J. Vet. Diagn. Invest.*, 4:127-133, 1992; Collins et al, Swine Infertility and Respiratory Syndrome/Mystery Swine Disease. *Proc., Minnesota Swine Conference for Veterinarians*, pp. 200-205, 1991), and in MARC-145 cells (Joo, PRRS: Diagnosis, *Proc., Allen D. Leman Swine Conference*, Veterinary Continuing Education and Extension,

University of Minnesota (1993), 20:53-55; Kim et al, *Arch. Virol.*, 133:477-483 (1993)). A successful culturing of a virus which causes SIRS has also been reported by Wensvoort et al (Mystery Swine Disease in the Netherlands: The
5 Isolation of Lelystad Virus. *Vet. Quart.* 13:121-130, 1991).

Initially, a number of agents were incriminated in the etiology of this disease (Wensvoort et al., *Vet. Res.* 24:117-124 (1993); Woolen et al., *J. Am. Vet. Med. Assoc.*
10 197:600-601 (1990)). There is now a consensus that the causative agent of PRRS is an enveloped RNA virus referred to as Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), reportedly of approximately 62 nm in diameter (Benfield et al., *J. Vet. Diagn. Invest.*, 4:127-133, 1992).

15 Virus isolates vary in their ability to replicate in continuous cell lines. Some grow readily, while others require several passages and some grow only in swine alveolar (SAM) cultures (Bautista et al., *J. Vet. Diagn. Invest.* 5:163-165, 1993; see also the Examples hereunder
20 [particularly Table 1]).

PRRSV is a member of an Arterivirus group which includes equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV) (Benfield et al., 1992, *supra*;
25 Plagemann, *Proc. Am. Assoc. Swine Practitioners*, 4:8-15 1992; Plagemann and Moennig, *Adv. Virus Res.* 41:99-192,

1992; Conzelmann et al., *Virology*, 193:329-339, 1993;
Godney et al., *Virology*, 194:585-596, 1993; Meulenberg et
al., *Virology*, 192:62-72, 1993). The positive-strand RNA
viruses of this Arterivirus group resemble togaviruses
5 morphologically, but are distantly related to coronaviruses
and toroviruses on the basis of genome organization and
gene expression (Plagemann et al., *supra*; Spaan et al., *J.*
Gen. Virol. 69, 2939-2952 (1988); Strauss et al., *Annu.*
Rev. Biochem. 42, 657-683 (1988); Lai, *Annu. Rev.*
10 *Microbiol.* 44, 303-333 (1990); Snijder et al., *Nucleic Acid*
Res. 18, 4535-4542 (1990)). The members of this group
infect macrophages and contain a nested set of 5 to 7
subgenomic mRNAs in infected cells (Plagemann et al.,
supra; Meulenberg et al., *Virology*, 192, 62-72 (1993);
15 Conzelmann et al., *Virology*, 193, 329-339 (1993); 15, 16,
17, 18, 19).

The viral genome of European isolates has been shown
to be a plus stranded RNA of about 15.1 kb (Conzelmann et
al., *supra*; Meulenberg et al., *supra*), and appears to be
20 similar in genomic organization to LDV and EAV (Meulenberg
et al., *supra*). However, no serological cross-reaction has
been found among PRRSV, LDV and EAV (Goyal et al., *J. Vet.*
Diagn. Invest., 5, 656-664 (1993)).

PRRSV was initially cultivated in swine alveolar
25 macrophage (SAM) cell cultures (Pol et al., *Veterinary*
Quarterly, 13:137-143, 1991; Wensvoort et al., *Veterinary*

Quarterly, 13:121-130, 1991) and then in continuous cell lines CL2621 (Benfield et al., *supra*), MA-104, and MARC-145 (Joo, *Proc. Allen D. Leman Swine Conference*, pp. 53-55, 1993). The reproductive and respiratory disease has been
5 reproduced with cell free lung filtrates (Christianson et al., *Am. J. Vet. Res.*, 53:485-488, 1992; Collins et al., *J. Vet. Diagn. Invest.*, 4:117-126, 1992; Halbur et al., *Proc. Central Veterinary Conference*, pp. 50-59, 1993), and with cell culture-propagated PRRSV (Collins et al., *supra*, and
10 *Proc. Allen D. Leman Swine Conference*, pp. 47-48, 1993).

Eight open reading frames (also referred to herein as "ORFs" or "genes") have been identified in a European PRRSV isolate. The genes of this European isolate are organized similarly to that in coronavirus (Meulenberg et al.,
15 *supra*). A 3'-end nested set of messenger RNA has been found in PRRSV-infected cells similar to that in coronaviruses (Conzelmann et al., *supra*; Meulenberg et al., *supra*).

The ORF 1a and 1b at the 5'-half of the European PRRSV
20 genome are predicted to encode viral RNA polymerase. The ORF's 2-6 at the 3'-half of the genome likely encode for viral membrane-associated (envelope) proteins (Meulenberg et al., *supra*). ORF6 is predicted to encode the membrane protein (M) based on its similar characteristics with the
25 ORF 6 of EAV, ORF 2 of LDV, and the M protein of mouse hepatitis virus and infectious bronchitis virus (Meulenberg

et al., *Virology* 192, 62-72 (1993); Conzelmann et al.,
Virology 193, 329-339 (1993); Murtaugh, *Proc. Allen D.*
Leman Swine Conference, Minneapolis, MN, pp. 43-45 (1993);
Mardassi et al., *Abstracts of Conference of Research*
5 *Workers in Animal Diseases*, Chicago, IL, pp. 43 (1993)).
The product of ORF 7 is extremely basic and hydrophilic,
and is predicted to be the viral nucleocapsid protein (N)
(Meulenberg et al., *supra*; Conzelmann et al., *supra*;
Murtaugh, *supra*; Mardassi et al., *supra* and *J. Gen. Virol.*,
10 75:681-685 (1994)).

Although conserved epitopes have been identified
between U.S. and European PRRSV isolates using monoclonal
antibodies (Nelson et al., *J. Clin. Microbiol.*,
31:3184-3189, 1993), there is extensive antigenic and
15 genetic variation both among U.S. and European isolates of
PRRSV (Wensvoort et al., *J. Vet. Diagn. Invest.*, 4:134-138,
1992). European isolates are genetically closely related,
as the nucleotide sequence at the 3'-half of the genome
from two European PRRSV isolates is almost identical
20 (Conzelmann et al., *supra*; Meulenberg et al., *supra*).

Although the syndrome caused by PRRSV appears to be
similar in the U.S. and Europe, several recent studies have
described phenotypic, antigenic, genetic and pathogenic
variations among PRRSV isolates in the U.S. and in Europe
25 (Murtaugh, *supra*; Bautista et al., *J. Vet. Diagn. Invest.*,
5, 163-165 (1993); Bautista et al., *J. Vet. Diagn. Invest.*,

5, 612-614 (1993); Wensvoort et al., *J. Vet. Diagn. Invest.*, 4, 134-138 (1992); Stevenson et al., *J. Vet. Diagn. Invest.*, 5, 432-434 (1993)). For example, the European isolates grow preferentially in SAM cultures and replicate to a very low titer in other culture systems (Wensvoort, *Vet. Res.*, 24, 117-124 (1993); Wensvoort et al., *J. Vet. Quart.*, 13, 121-130 (1991); Wensvoort et al., *J. Vet. Diagn. Invest.*, 4, 134-138 (1992)). On the other hand, some of the U.S. isolates have been shown to replicate well in SAM as well as in the continuous cell line CL2621 (Benfield et al., *J. Vet. Diagn. Invest.*, 4, 127-133 (1992); Collins et al., *J. Vet. Diagn. Invest.*, 4, 117-126 (1992)). Thus, phenotypic differences among U.S. isolates are observed, as not all PRRSV isolates isolated on SAM can replicate on the CL2621 cell line (Bautista et al., *J. Vet. Diagn. Invest.*, 5, 163-165 (1993)).

A high degree of regional antigenic variation among PRRSV isolates may exist. Four European isolates were found to be closely related antigenically, but these European isolates differed antigenically from U.S. isolates. Further, three U.S. isolates were shown to differ antigenically from each other (Wensvoort et al., *J. Vet. Diagn. Invest.*, 4, 134-138 (1992)). Animals seropositive for European isolates were found to be negative for U.S. isolate VR 2332 (Bautista et al., *J. Vet. Diagn. Invest.*, 5, 612-614 (1993)).

U.S. PRRSV isolates differ genetically at least in part from European isolates (Conzelmann et al., *supra*; Meulenberg et al., *supra*; Murtaugh et al., *Proc. Allen D. Lemman Conference*, pp. 43-45, 1993). The genetic

5 differences between U.S. and European isolates are striking, especially since they are considered to be the same virus (Murtaugh, *supra*). Similar observations were also reported when comparing the Canadian isolate IAF-exp91 and another U.S. isolate VR 2332 with LV (Murtaugh, *supra*;
10 Mardassi, *supra*). However, the 3' terminal 5 kb nucleotide sequences of two European isolates are almost identical (Conzelmann et al., *supra*; Meulenberg et al., *supra*).

The existence of apathogenic or low-pathogenic strains among isolates has also been suggested (Stevenson, *supra*).
15 Thus, these studies suggest that the PRRSV isolates in North America and in Europe are antigenically and genetically heterogeneous, and that different genotypes or serotypes of PRRSV exist. However, prior to the present invention, the role of antigenic and genetic variation in
20 the pathogenesis of PRRSV was not entirely clear.

The occurrence of PRRS in the U.S. has adversely affected the pig farming industry. Almost half of swine herds in swine-producing states in the U.S. are seropositive for PRRSV (*Animal Pharm.*, 264:11 (11/11/92)).
25 In Canada, PRRS has been characterized by anorexia and pyrexia in sows lasting up to 2 weeks, late-term abortions,

increased stillbirth rates, weak-born pigs and neonatal deaths preceded by rapid abdominal breathing and diarrhea. Work on the isolation of the virus causing PRRS, on a method of diagnosing PRRS infection, and on the development of a vaccine against the PRRS virus has been published (see Canadian Patent Publication No. 2,076,744; PCT International Patent Publication No. WO 93/03760; PCT International Patent Publication No. WO 93/06211; and PCT International Patent Publication No. WO 93/07898).

There is also variability in the virulence of PRRSV in herds. Recently, a more virulent form of PRRS has been occurring with increased incidence in 3-8 week old pigs in the midwestern United States. Typically, healthy 3-5 week old pigs are weaned and become sick 5-7 days later.

Routine virus identification methods on tissues from affected pigs have shown that swine influenza virus (SIV), pseudorabies virus (PRV), and Mycoplasma hyopneumoniae are not associated with this new form of PRRS. Originally termed proliferative interstitial pneumonia (PIP; see U.S. patent application Serial No. 07/969,071), this disease has been very recently linked with PRRS, and the virus has been informally named the "Iowa strain" of PRRSV (see U.S. patent application Serial No. 08/131,625).

Pessimism and skepticism has been expressed in the art concerning the development of effective vaccines against these porcine viruses (*The Veterinary Record*, October 26,

1991). A belief that human influenza vaccine may afford some protection against the effects of PRRS and PNP exists (*The Veterinary Record*, July 6, 1991).

Viral envelope proteins are known to be highly
5 variable in many coronaviruses, such as feline infectious
peritonitis virus and mouse hepatitis virus (Dalziel et al:
Site-specific alteration of murine hepatitis virus type 4
peplomer glycoprotein E2 results in reduced neurovirulence.
J. Virol., 59:464-471 (1986); Fleming et al: Pathogenicity
10 of antigenic variants of murine coronavirus JHM selected
with monoclonal antibodies. *J. Virol.*, 58:869-875 (1986);
Fiscus et al: Antigenic comparison of the feline
coronavirus isolates; Evidence for markedly different
peplomer glycoproteins. *J. Virol.*, 61:2607-2613 (1987);
15 Parker et al: Sequence analysis reveals extensive
polymorphism and evidence of deletions within the E2
glycoprotein gene of several strains of murine hepatitis
virus. *Virology*, 173:664-673 (1989)).

For example, a deletion or a mutation in the major
20 envelope protein in coronaviruses can alter tissue tropism
and *in vivo* pathogenicity. A mutation in a monoclonal
antibody-resistant mutant of MHV has resulted in loss of
its neurovirulence for mice (Fleming et al, 1986 *supra*).
Porcine respiratory coronavirus (PRCV) is believed to be a
25 deletion mutant of transmissible gastroenteritis virus
(TGEV) in swine. The deletion in the PRCV genome may be in

the 5'-end of the spike (S) gene of TGEV (Halbur et al, An
overview of porcine viral respiratory disease. *Proc.*
Central Veterinary Conference, pp. 50-59 (1993); Laude et
al, Porcine respiratory coronavirus: Molecular features
5 and virus-host interactions. *Vet. Res.*, 24:125-150 (1993);
Vaughn et al, Isolation and characterization of three
porcine respiratory coronavirus isolates with varying sizes
of deletions. *J. Clin. Micro.*, 32:1809-1812 (1994)).

PRCV has a selective tropism for the respiratory tract
10 and does not replicate in the gastrointestinal tract
(Rasschaert et al, Porcine respiratory coronavirus differs
from transmissible gastroenteritis virus by a few genomic
deletions. *J. Gen. Virol.*, 71:2599-2607 (1990); Laude et
al, 1993 *supra*). In contrast, TGEV has a tropism for both
15 respiratory and gastrointestinal tracts (Laude et al, 1993
supra).

Variation in antigenic and genetic relatedness among
LDV isolates of varying pathogenicity is also known (Kuo et
al, Lactate-dehydrogenase-elevating virus (LDV):
20 subgenomic mRNAs, mRNA leader and comparison of 3'-terminal
sequences of two LDV isolates. *Virus Res.*, 23:55-72
(1992); Plagemann, LDV, EAV, and SHFV: A new group of
positive stranded RNA viruses. *Proc. Am. Assoc. Swine*
Practitioners, 4:8-15 (1992); Chen et al, Sequences of 3'
25 end of genome and of 5' end of open reading frame 1a of
lactate dehydrogenase-elevating virus and common junction

motifs between 5' leader and bodies of seven subgenomic mRNAs. *J. Gen. Virol.*, 74:643-660 (1993)).

However, the present invention provides the first insight into the relationships between the open reading
5 frames of the PRRSV genome and their corresponding effects on virulence and replication.

Further, a diagnosis of porcine reproductive and respiratory syndrome (PRRS) relies on compiling information from the clinical history of the herd, serology, pathology,
10 and ultimately on isolation of the PRRS virus (PRRSV). Three excellent references reviewing diagnosis of PRRSV have been published in the last year (Van Alstine et al, "Diagnosis of porcine reproductive and respiratory syndrome," *Swine Health and Production*, Vol. 1, No. 4
15 (1993), p. 24-28; Christianson et al, "Porcine reproductive and respiratory syndrome: A review." *Swine Health and Production*, Vol. 1, No. 2 (1994), pp. 10-28 and Goyal, "Porcine reproductive and respiratory syndrome," *J. Vet. Diagn. Invest.* 5:656-664 (1993)). PRRSV has also recently
20 been shown to replicate in pulmonary alveolar macrophages by gold colloid immunohistochemistry (Magar et al (1993): Immunohistochemical detection of porcine reproductive and respiratory syndrome virus using colloidal gold. *Can. J. Vet. Res.*, 57:300-304).

25 Clinical signs vary widely between farms, and thus, are not the most reliable evidence of a definitive

diagnosis, except in the case of a severe acute outbreak in naive herds which experience abortion storms, increased numbers of stillborn pigs, and severe neonatal and nursery pig pneumonia. Presently, the most common clinical
5 presentation is pneumonia and miscellaneous bacterial problems in 3-10 week old pigs. However, many PRRSV-positive herds have no apparent reproductive or respiratory problems.

Some herds evidence devastating reproductive failure,
10 characterized by third-trimester abortions, stillborn pigs and weak-born pigs. Many of these herds also experience severe neonatal respiratory disease. Respiratory disease induced by PRRSV in 4-10 week-old pigs is also common and can be quite severe (Halbur et al, Viral contributions to
15 the porcine respiratory disease complex. Proc. Am. Assoc. Swine Pract. (1993), pp. 343-350). Clinical PRRSV outbreaks are frequently followed by bacterial pneumonia, septicemia, or enteritis. Thus, it has been difficult to obtain an acceptably rapid and reliable diagnosis of
20 infection by PRRSV, prior to the present invention.

The pig farming industry has been and will continue to be adversely affected by these porcine reproductive and respiratory diseases and new variants thereof, as they appear. PRRSV is a pathogen of swine that causes economic
25 losses from reproductive and respiratory diseases. Economic losses from PRRS occur from loss of pigs from

abortions, stillborn pigs, repeat breeding, pre-weaning and postweaning mortality, reduced feed conversion efficiency, increased drug and labor cost and have been estimated to cost approximately \$236 per sow in addition to loss of profits (Polson et al.; Financial implications of mystery swine disease (MSD), *Proc. Mystery Swine Disease Committee Meeting*, Denver, Co., 1990, pp. 8-28). This represents a loss of \$23,600 for a 100 sow herd or \$236,000 for a 1000 sow herd.

PRRSV causes additional losses from pneumonia in nursery pigs. However, the exact economic losses from PRRSV-associated pneumonia are not known. PRRSV is an important cause of pneumonia in nursery and weaned pigs. Reproductive disease was the predominant clinical outcome of PRRSV infections during the past few years. Respiratory disease has now become the main problem associated with PRRSV.

Surprisingly, the market for animal vaccines in the U.S. and worldwide is larger than the market for human vaccines. Thus, there exists an economic incentive to develop new veterinary vaccines, in addition to the substantial public health benefit which is derived from protecting farm animals from disease.

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide a polynucleic acid isolated from a porcine reproductive and respiratory virus (PRRSV).

5 It is a further object of the present invention to provide an isolated polynucleic acid which encodes a PRRSV protein.

 It is a further object of the present invention to provide a PRRSV protein, either isolated from a PRRSV or
10 encoded by a PRRSV polynucleic acid.

 It is a further object of the present invention to provide a protein- or polynucleic acid-based vaccine which protects a pig against PRRS.

 It is a further object of the present invention to
15 provide a method of raising an effective immunological response against a PRRSV using the vaccine.

 It is a further object of the present invention to provide a method of producing a protein- or polynucleic acid-based vaccine which protects a pig against a PRRSV
20 infection.

 It is a further object of the present invention to provide a method of treating a pig infected by or exposed to a PRRSV.

 It is a further object of the present invention to
25 provide a method of detecting PRRSV.

It is a further object of the present invention to provide an immunoperoxidase diagnostic assay for detection of PRRSV antigen in porcine tissues.

5 It is a further object of the present invention to provide an antibody which immunologically binds to a PRRSV protein or to an antigenic region of such a protein.

It is a further object of the present invention to provide an antibody which immunologically binds to a protein- or polynucleic acid-based vaccine which protects a
10 pig against a PRRSV.

It is a further object of the present invention to provide a method of treating a pig exposed to or infected by a PRRSV.

It is a further object of the present invention to
15 provide a method of detecting and a diagnostic kit for assaying a PRRSV.

It is a further object of the present invention to provide the above objects, where the PRRS virus is the Iowa strain of PRRSV.

20 These and other objects which will become apparent during the following description of the preferred embodiments, have been provided by at least one purified polypeptide selected from the group consisting of proteins encoded by one or more open reading frames (ORF's) of an
25 Iowa strain of porcine reproductive and respiratory virus (PRRSV), proteins at least 80% but less than 100%

homologous with those encoded by one or more of ORF 2, ORF 3, ORF 4 and ORF 5 of an Iowa strain of PRRSV, proteins at least 97% but less than 100% homologous with proteins encoded by one or both of ORF 6 and ORF 7 of an Iowa strain of PRRSV, antigenic regions of said proteins which are at least 5 amino acids in length and which effectively stimulate immunological protection in a porcine host against a subsequent challenge with a PRRSV isolate, and combinations thereof; an isolated polynucleic acid which encodes such a polypeptide or polypeptides; a vaccine comprising an effective amount of such a polynucleotide or polypeptide(s); antibodies which specifically bind to such a polynucleotide or polypeptide; methods of producing the same; and methods of raising an effective immunological response against a PRRSV, treating a pig exposed to or infected by a PRRSV, and detecting a PRRSV using the same.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a flowchart outlining a procedure for producing a subunit vaccine;

Figure 2 is a flowchart outlining a procedure for producing a genetically engineered vaccine;

Figure 3 shows a general schematic procedure for the construction of a cDNA λ library as described by the manufacturer (Stratagene);

Figure 4 shows a general schematic procedure for identifying authentic clones of the PRRS virus isolate ISU-12 (VR 2385) by differential hybridization (modified from "Recombinant DNA," 2nd ed., Watson, J.D., et al., eds. (1992), p. 110);

Figure 5 is a Northern blot showing the VR 2385 subgenomic mRNA species, denatured with 6 M glyoxal and DMSO, and separated on a 1.5% agarose gel;

Figure 6 shows the λ cDNA clones used to obtain the 3'-terminal nucleotide sequence of VR 2385;

Figure 7 shows the 2062-bp 3'-terminal sequence (SEQ ID NO:13) and the amino acid sequences encoded by ORF's 5, 6 and 7 (SEQ ID NOS:15, 17 and 19, respectively) of VR 2385;

Figure 8 compares the ORF-5 regions of the genomes of VR 2385 and Lelystad virus;

Figure 9 compares the ORF-6 regions of the genomes of VR 2385 and Lelystad virus;

Figure 10 compares the ORF-7 regions of the genomes of VR 2385 and Lelystad virus;

Figure 11 compares the 3'-nontranslational regions of the genomes of VR 2385 and Lelystad virus;

Figure 12 shows a cytopathic effect in HI-FIVE cells infected with a recombinant baculovirus containing the VR 2385 ORF-7 gene (Baculo.PRRSV.7);

Figure 13 shows HI-FIVE cells infected with a recombinant baculovirus containing the VR 2385 ORF-6 gene, stained with swine antisera to VR 2385, followed by fluorescein-conjugated anti-swine IgG;

5 Figure 14 shows HI-FIVE cells infected with a recombinant baculovirus containing the VR 2385 ORF-7 gene, respectively, stained with swine antisera to VR 2385, followed by fluorescein-conjugated anti-swine IgG;

10 Figure 15 shows a band of expected size for the VR 2385 ORF-6 product, detected by a radioimmunoprecipitation technique (see Experiment II(B) below);

Figure 16 shows a band of expected size for the VR 2385 ORF-7 product, detected by a radioimmunoprecipitation technique (see Experiment II(B) below);

15 Figure 17 compares the ORF 6 and ORF 7 nucleotide sequences of six U.S. PRRSV isolates and of LV, in which the VR 2385 nucleotide sequence is shown first, and in subsequent sequences, only those nucleotides which are different are indicated;

20 Figures 18(A) - (B) show the alignment of amino acid sequences of the putative M (Fig. 18(A)) and N (Fig. 18(B)) genes of the proposed arterivirus group, performed with a GENEWORKS program (IntelliGenetics, Inc.);

25 Figures 19(A) - (B) show phylogenetic trees based on the amino acid sequences of the putative M (Fig. 19(A)) and N genes (Fig. 19(B)) for the proposed arterivirus group;

Figure 20 shows the nucleotide sequence of a region of the genome of PRRSV isolate VR 2385 containing ORF's 2, 3 and 4;

Figures 21(A) - (C) compare the nucleotide sequences of ORF 2, ORF 3 and ORF 4 of PRRSV VR 2385 with the corresponding ORF's of Lelystad virus (LV);

Figures 22(A) - (C) show alignments of the predicted amino acid sequences encoded by ORF's 2, 3 and 4 of PRRSV VR 2385 and LV;

Figure 23 shows an immunohistochemical stain of a lung tissue sample taken from a pig infected 9 days previously with PRRSV, in which positive ABC staining with hematoxylin counterstain is observed within the cytoplasm of macrophages and sloughed cells in the alveolar spaces;

Figure 24 shows an immunohistochemical stain of a lung tissue sample taken from a pig infected 4 days previously with PRRSV, in which positive ABC staining with hematoxylin counterstain is demonstrated within cellular debris in terminal airway lumina;

Figure 25 shows a heart from a pig infected 9 days previously with PRRSV, in which positive staining is demonstrated within endothelial cells (arrow) and isolated macrophages by the present streptavidin-biotin complex method (with hematoxylin counterstain); the bar indicates a length of 21 microns;

Figure 26 shows a tonsil from a pig infected 9 days previously with PRRSV, in which positive staining cells (arrow heads) are demonstrated within follicles and in the crypt epithelium by the present streptavidin-biotin complex method (with hematoxylin counterstain); the bar indicates a length of 86 microns;

Figure 27 shows a lymph node from a pig infected 9 days previously with PRRSV, in which positive staining is demonstrated within follicles by the present streptavidin-biotin complex method (with hematoxylin counterstain), and positive cells (arrows) resemble macrophages or dendritic cells; the bar indicates a length of 21 microns;

Figures 28(A)-(C) are photomicrographs of lungs from pig inoculated with (A) culture fluid from an uninfected cell line, (B) culture fluid from a cell line infected with a low virulence PRRSV isolate (the lungs show PRRS-A type lesions), and (C) culture fluid from a cell line infected with a high virulence PRRSV isolate (the lungs show PRRS-B type lesions);

Figures 29(A)-(B) illustrate immunohistochemical staining with anti-PRRSV monoclonal antibody of a lung from a pig infected 9 days previously with PRRSV; and

Figures 30(A)-(B) show Northern blots of PRRSV isolates VR 2385pp (designated as "12"), VR 2429 (ISU-22, designated as "22"), VR 2430, designated as "55"), ISU-79

(designated as "79"), ISU-1894 (designated as "1894"), and VR 2431, designated as "3927").

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the present invention, a "porcine reproductive and
5 respiratory syndrome virus" or "PRRSV" refers to a virus
which causes the diseases PRRS, PEARS, SIRS, MSD and/or PIP
(the term "PIP" now appears to be disfavored), including
the Iowa strain of PRRSV, other strains of PRRSV found in
the United States (e.g., VR 2332), strains of PRRSV found
10 in Canada (e.g., IAF-exp91), strains of PRRSV found in
Europe (e.g., Lelystad virus, PRRSV-10), and closely-
related variants of these viruses which may have appeared
and which will appear in the future.

The present vaccine is effective if it protects a pig
15 against infection by a porcine reproductive and respiratory
syndrome virus (PRRSV). A vaccine protects a pig against
infection by a PRRSV if, after administration of the
vaccine to one or more unaffected pigs, a subsequent
challenge with a biologically pure virus isolate (e.g., VR
20 2385, VR 2386, or other virus isolate described below)
results in a lessened severity of any gross or
histopathological changes (e.g., lesions in the lung)
and/or of symptoms of the disease, as compared to those
changes or symptoms typically caused by the isolate in
25 similar pigs which are unprotected (i.e., relative to an

appropriate control). More particularly, the present vaccine may be shown to be effective by administering the vaccine to one or more suitable pigs in need thereof, then after an appropriate length of time (e.g., 1-4 weeks),
5 challenging with a large sample (10^{3-7} TCID₅₀) of a biologically pure PRRSV isolate. A blood sample is then drawn from the challenged pig after about one week, and an attempt to isolate the virus from the blood sample is then performed (e.g., see the virus isolation procedure
10 exemplified in Experiment VIII below). Isolation of the virus is an indication that the vaccine may not be effective, and failure to isolate the virus is an indication that the vaccine may be effective.

Thus, the effectiveness of the present vaccine may be
15 evaluated quantitatively (i.e., a decrease in the percentage of consolidated lung tissue as compared to an appropriate control group) or qualitatively (e.g., isolation of PRRSV from blood, detection of PRRSV antigen in a lung, tonsil or lymph node tissue sample by an
20 immunoperoxidase assay method [described below], etc.). The symptoms of the porcine reproductive and respiratory disease may be evaluated quantitatively (e.g., temperature/fever), semi-quantitatively (e.g., severity of respiratory distress [explained in detail below], or qualitatively
25 (e.g., the presence or absence of one or more symptoms or a

reduction in severity of one or more symptoms, such as cyanosis, pneumonia, heart and/or brain lesions, etc.).

5 An unaffected pig is a pig which has either not been exposed to a porcine reproductive and respiratory disease infectious agent, or which has been exposed to a porcine reproductive and respiratory disease infectious agent but is not showing symptoms of the disease. An affected pig is one which shows symptoms of PRRS or from which PRRSV can be isolated.

10 The clinical signs or symptoms of PRRS may include lethargy, respiratory distress, "thumping" (forced expiration), fevers, roughened haircoats, sneezing, coughing, eye edema and occasionally conjunctivitis. Lesions may include gross and/or microscopic lung lesions, myocarditis, lymphadenitis, encephalitis and rhinitis. The
15 infectious agent may be a single virus, or may be combined with one or more additional infectious agents (e.g., other viruses or bacteria). In addition, less virulent and non-virulent forms of the PRRSV and of Iowa strain have been
20 found, which may cause either a subset of the above symptoms or no symptoms at all. Less virulent and non-virulent forms of PRRSV can be used according to the present invention to provide protection against porcine reproductive and respiratory diseases nonetheless.

25 Histological lesions in the various porcine diseases are different. Table I below compares physiological

observations and pathology of the lesions associated with a number of diseases caused by porcine viruses:

TABLE I

Swine Viral Pneumonia Comparative Pathology

Lesion	PRRS(p)	PRRS(o)	SIV	PNP	PRCV	PPMV	Iowa
Type II	+	+++	+	+++	++	++	++++
Inter. thickening	++++	+	+	+	++	++	+
Alveolar exudate	+	+++	++	++	++	++	+++
Airway necrosis	-	-	++++	++++	+++	+	-
Syncytia	-	++	+/-	++	+	+	+++
Encephalitis	+	+++	-	-	-	++	+
Myocarditis	+/-	++	-	-	-	-	+++

wherein "PRRS(p)" represents the published pathology of the
5 PRRS virus, "PRRS(o)" represents the pathology of PRRS
virus observed by the present Inventors, "SIV" represents
swine influenza A virus, "PRCV" represents porcine
respiratory coronavirus, "PPMV" represents porcine
paramyxovirus, "Iowa" refers to the strain of PRRSV
10 discovered by the present Inventors, "Type II" refers to
Type II pneumocytes (which proliferate in infected pigs),
"Inter." refers to interstitial septal infiltration by
mononuclear cells, "Airway necrosis" refers to necrosis in
terminal airways, and the symbols (-) and (+) through
15 (++++), refer to a comparative severity scale as follows:

(-): negative (not observed)

(+): mild (just above the threshold of observation)

(++): moderate

(+++): severe

5 (++++): most severe

A "porcine reproductive and respiratory virus" or "PRRSV" causes a porcine reproductive and respiratory disease defined by one or more of the clinical signs, symptoms, lesions and histopathology as described above, and is characterized as being an enveloped RNA arterivirus, 10 having a size of from 50 to 80 nm in diameter and from 250 to 400 nm in length. "North American strains of PRRSV" refer to those strains of PRRSV which are native to North America. "U.S. strains of PRRSV" refer to strains of PRRSV 15 native to the U.S., and "European strains of PRRSV" refer to strains native to Europe, such as Lelystad virus (deposited by the CDI [Lelystad, Netherlands] in the depository at the Institut Pasteur, Paris, France, under the deposit number I-1102; see International Patent 20 Publication No. WO 92/21375, published on December 10, 1992).

The "Iowa strain" of PRRSV refers to (a) those strains of PRRSV isolated by the presented Inventors, (b) those strains having at least a 97% sequence identity (or 25 homology) in the seventh open reading frame (ORF 7) with at least one of VR 2385, VR 2430 and VR 2431; (c) strains

which, after no more than 5 passages, grow to a titer of at least 10^4 TCID₅₀ in CRL 11171 cells, MA-104 cells or PSP-36 cells, (d) those strains having at least 80% and preferably at least 90% homology with one or more of ORF's 2-5 of VR 2385, and (e) those strains which cause a greater percentage consolidation of lung tissue than Lelystad virus (e.g., at 10 days post-infection, infected pigs exhibit at least 20% and preferably at least 40% lung consolidation). Preferably, the Iowa strain of PRRSV is characterized by at least two of the above characteristics (a)-(e).

The present invention is primarily concerned with polynucleic acids (segments of genomic RNA and/or DNA, mRNA, cDNA, etc.) isolated from or corresponding to a porcine reproductive and respiratory syndrome virus (PRRSV), proteins encoded by the DNA, methods of producing the polynucleic acids and proteins, vaccines which protect pigs from a PRRSV, a method of protecting a pig from a PRRSV using the vaccine, a method of producing the vaccine, a method of treating a pig infected by or exposed to a PRRSV, and a method of detecting a PRRSV. More particularly, the present invention is concerned with a vaccine which protects pigs from North American strains of PRRSV, a method of producing and administering the vaccine, and polynucleic acids and proteins obtained from an Iowa strain of PRRSV. However, it is believed that the information learned in the course of developing the present

invention will be useful in developing vaccines and methods of protecting pigs against any and/or all strains of porcine reproductive and respiratory syndrome. Therefore, the present invention is not necessarily limited to
5 polynucleic acids, proteins, vaccines and methods related to the Iowa strain of PRRS virus (PRRSV).

The phrase "polynucleic acid" refers to RNA or DNA, as well as mRNA and cDNA corresponding to or complementary to the RNA or DNA isolated from the virus or infectious agent.
10 An "ORF" refers to an open reading frame, or polypeptide-encoding segment, isolated from a viral genome, including the PRRSV genome. In the present polynucleic acid, an ORF can be included in part (as a fragment) or in whole, and can overlap with the 5'- or 3'-sequence of an adjacent ORF
15 (see Figs. 7 and 21, and Experiments I and IV below). A "polynucleotide" is equivalent to a polynucleic acid, but may define a distinct molecule or group of molecules (e.g., as a subset of a group of polynucleic acids).

Referring now to Figures 1-2, flowcharts of procedures
20 are provided for preparing types of vaccines encompassed by the present invention. The flowcharts of Figures 1-2 are provided as exemplary methods of producing the present vaccines, and are not intended to limit the present invention in any manner.

25 The first step in each procedure detailed in Figures 1-2 is to identify a cell line susceptible to infection

with a porcine reproductive and respiratory virus or infectious agent. (To simplify the discussion concerning preparation of the vaccine, the term "virus" refers to a virus and/or other infectious agent associated with a porcine reproductive and respiratory disease.) A master cell stock (MCS) of the susceptible host cell is then prepared. The susceptible host cells continue to be passaged beyond MCS. Working cell stock (WCS) is prepared from cell passages between MCS and MCS+n.

10 A master seed virus is propagated on the susceptible host cell line, between MCS and MCS+n, preferably on WCS. The raw virus is isolated by methods known in the art from appropriate, preferably homogenized, tissue samples taken from infected pigs exhibiting disease symptoms
15 corresponding to those caused by the virus of interest. A suitable host cell, preferably a sample of the WCS, is infected with the raw virus, then cultured. Vaccine virus is subsequently isolated and plaque-purified from the infected, cultured host cell by methods known in the art.
20 Preferably, the virus to be used to prepare the vaccine is plaque-purified three times.

Master seed virus (MSV) is then prepared from the plaque-purified virus by methods known in the art. The MSV(X) is then passaged in WCS at least four times through
25 MSV(X+1), MSV(X+2), MSV(X+3) and MSV(X+4) virus passages. The MSV(X+4) is considered to be the working seed virus.

Preferably, the virus passage to be used in the pig studies and vaccine product of the present invention is MSV(X+5), the product of the fifth passage.

In conjunction with the working cell stock, the
5 working seed virus is cultured by known methods in
sufficient amounts to prepare a prototype vaccine,
preferably MSV(X+5). The present prototype vaccines may be
of any type suitable for use in the veterinary medicine
field. The primary types of vaccines on which the present
10 invention focuses include a subunit vaccine (Figure 1) and
a genetically engineered vaccine (Figure 2). However,
other types of vaccines recognized in the field of
veterinary vaccines, including live, modified live,
attenuated and killed virus vaccines, are also acceptable.
15 A killed vaccine may be rendered inactive through chemical
treatment or heat, etc., in a manner known to the artisan
of ordinary skill.

An attenuated virus may be obtained by repeating
serial passage of the virus in a suitable host cell a
20 sufficient number of times to obtain an essentially non-
virulent virus. For example, a PRRSV may be serially
passaged from 1 to 20 times (or more, if desired), in order
to render it sufficiently attenuated for use as an
attenuated vaccine. MSV(X+5) may be such an attenuated
25 vaccine.

In the procedures outlined by each of Figures 1-2, following preparation of a prototype vaccine, pig challenge models and clinical assays are conducted by methods known in the art. For example, before performing actual
5 vaccination/challenge studies, the disease to be prevented and/or treated must be defined in terms of its symptoms, clinical assay results, conditions, etc. As described herein, the Iowa strain of PRRSV has been defined in terms of its histopathology and the clinical symptoms which it
10 causes. Clinical analyses of the Iowa strain of PRRSV are described in detail in the Experiments below.

One then administers a prototype vaccine to a pig, then exposes the pig to the virus which causes the disease. This is known as "challenging" the pig and its
15 immunological system. After observing the response of the challenged pig to exposure to the virus or infectious agent and analyzing the ability of the prototype vaccine to protect the pig, efficacy studies are then performed by conventional, known methods. A potency assay is then
20 developed in a separate procedure by methods known in the art, and prelicensing serials are then produced.

Prior to preparation of the prototype subunit vaccine (Figure 1), the protective or antigenic components of the vaccine virus should be identified. Such protective or
25 antigenic components include certain amino acid segments or fragments of the viral proteins (preferably coat proteins)

which raise a particularly strong protective or immunological response in pigs; such antigenic protein fragments fused to non-PRRSV proteins which act as a carrier and/or adjuvant; single or multiple viral coat proteins themselves, oligomers thereof, and higher-order associations of the viral coat proteins which form virus substructures or identifiable parts or units of such substructures; oligoglycosides, glycolipids or glycoproteins present on or near the surface of the virus or in viral substructures such as the nucleocapsid; lipoproteins or lipid groups associated with the virus, etc.

Antigenic amino acid segments or fragments are preferably at least 5 amino acids in length, particularly preferably at least 10 amino acids in length, and can be up to but not including the entire length of the native protein. In the present invention, the binding affinity (or binding constant or association constant) of an antigenic fragment is preferably at least 1% and more preferably at least 10% of the binding affinity of the corresponding full-length protein (i.e., which is encoded by the same ORF) to a monoclonal antibody which specifically binds the full-length protein. The monoclonal antibody which specifically binds to the full-length protein encoded by an ORF of a PRRSV is preferably deposited under the Budapest Treaty at an acceptable

depository, or is sequenced or otherwise characterized in terms of its physicochemical properties (e.g., antibody type [IgG, IgM, etc.], molecular weight, number of heavy and light chains, binding affinities to one or more known
5 or sequenced proteins [e.g., selected from SEQ ID NOS:15, 17, 19, 21, 24, 26, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 67, 69, 71, 73, 75 and 77], etc.).

Antigenic fragments of viral proteins (e.g., those encoded by one or more of ORF's 2-6 of a PRRSV virus) are
10 identified by methods known in the art. For example, one can prepare polynucleic acids having a truncated ORF encoding a polypeptide with a predetermined number of amino acid residues deleted from the N-terminus, C-terminus, or both. The truncated ORF can be expressed *in vitro* or *in*
15 *vivo* in accordance with known methods, and the corresponding truncated polypeptide can then be isolated in accordance with known methods. The immunoprotective properties of the polypeptides may be measured directly (e.g., *in vivo*). Alternatively, the antigenic region(s) of
20 the full-length polypeptide can be determined indirectly by screening a series of truncated polypeptides against, for example, suitably deposited or characterized monoclonal antibodies. (If the alternative, indirect method is performed, the failure of a truncated polypeptide to bind
25 to a neutralizing monoclonal antibody is a strong indication that the portion of the full-length polypeptide

deleted in the truncated polypeptide contains an antigenic fragment.) Once identified, the antigenic or immunoprotective portion(s) (the "subunit(s)") of the viral proteins or of the virus itself may be subsequently cloned and/or purified in accordance with known methods. (The viral/bacterial inactivation and subunit purification protocols recited in Fig. 1 are optional.)

Genetically engineered vaccines (Figure 2) begin with a modification of the general procedure used for preparation of the other vaccines. After plaque-purification, the PRRS virus may be isolated from a suitable tissue homogenate by methods known in the art, preferably by conventional cell culture methods using PSP-36, ATCC CRL 11171 or macrophage cells as hosts.

The RNA is extracted from the biologically pure virus by a known method, preferably by the guanidine isothiocyanate method using a commercially available RNA isolation kit (for example, the kit available from Stratagene, La Jolla, California), and purified by one or more known methods, preferably by ultracentrifugation in a CsCl gradient. Messenger RNA may be further purified or enriched by oligo (dT)-cellulose column chromatography.

The viral genome is then cloned into a suitable host by methods known in the art (see Maniatis et al, "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory (1989), Cold Spring Harbor, Massachusetts). The

virus genome is then analyzed to determine essential regions of the genome for producing antigenic portions of the virus. Thereafter, the procedure for producing a genetically engineered vaccine is essentially the same as
5 for a modified live vaccine, an inactivated vaccine or a subunit vaccine (see Figure 1 of the present application and Figures 1-3 of U.S. application Serial No. 08/131,625). During prelicensing serials, expression of the cloned, recombinant subunit of a subunit vaccine may be optimized
10 by methods known to those in the art (see, for example, relevant sections of Maniatis et al, cited above).

The present vaccine protects pigs against a virus or infectious agent which causes a porcine reproductive and respiratory disease. Preferably, the present vaccine
15 protects pigs against infection by PRRSV. However, the present vaccine is also expected to protect a pig against infection by closely related variants of various strains of PRRSV as well.

Subunit virus vaccines may also be prepared from semi-
20 purified virus subunits by the methods described above in the discussion of Figure 1. For example, hemagglutinin isolated from influenza virus and neuraminidase surface antigens isolated from influenza virus have been prepared, and shown to be less toxic than the whole virus. Subunit
25 vaccines can also be prepared from highly purified subunits of the virus. An example in humans is the 22-nm surface

antigen of human hepatitis B virus. Human herpes simplex virus subunits and many other examples of subunit vaccines for use in humans are known. Thus, methods of preparing purified subunit vaccines from PRRSV cultured in a suitable host cell may be applicable to the present subunit vaccine.

Attenuated virus vaccines can be found in nature and may have naturally-occurring gene deletions (see Experiments VIII and IX below). Alternatively, attenuated vaccines may be prepared by a variety of known methods, such as serial passage (e.g., 5-25 times) in cell cultures or tissue cultures. However, the attenuated virus vaccines preferred in the present invention are those attenuated by recombinant gene deletions or gene mutations (as described above).

Genetically engineered vaccines are produced by techniques known to those in the art. Such techniques include those using recombinant DNA and those using live viruses. For example, certain virus genes can be identified which code for proteins responsible for inducing a stronger immune or protective response in pigs. Such identified genes can be cloned into protein expression vectors, such (but not limited to) as the baculovirus vector (see, for example, O'Reilly et al, "Baculovirus Expression Vectors: A Lab Manual," Freeman & Co. (1992)). The expression vector containing the gene encoding the immunogenic virus protein can be used to infect appropriate

host cells. The host cells are cultured, thus expressing the desired vaccine proteins, which can be purified to a desired extent, then used to protect the pigs from a reproductive and respiratory disease.

5 Genetically engineered proteins may be expressed, for example, in insect cells, yeast cells or mammalian cells. The genetically engineered proteins, which may be purified and/or isolated by conventional methods, can be directly inoculated into animals to confer protection against
10 porcine reproductive and respiratory diseases. One or more envelope proteins from a PRRSV (i.e., those encoded by ORF's 2-6) or antigenic portions thereof may be used in a vaccine to induce neutralizing antibodies. Nucleoproteins from a PRRSV may be used in a vaccine to induce cellular
15 immunity.

 Preferably, the present invention transforms an insect cell line (HI-FIVE) with a transfer vector containing polynucleic acids obtained from the Iowa strain of PRRSV. Preferably, the present transfer vector comprises
20 linearized baculovirus DNA and a plasmid containing one or more polynucleic acids obtained from the Iowa strain of PRRSV. The host cell line may be co-transfected with the linearized baculovirus DNA and a plasmid, so that a recombinant baculovirus is made. Particularly preferably,
25 the present polynucleic acid encodes one or more proteins of the Iowa strain of PRRSV.

Alternatively, RNA or DNA from a PRRSV encoding one or more viral proteins (e.g., envelope and/or nucleoproteins) can be inserted into live vectors, such as a poxvirus or an adenovirus, and used as a vaccine.

5 Thus, the present invention further concerns a purified preparation of a polynucleic acid isolated from the genome of a PRRS virus, preferably a polynucleic acid isolated from the genome of the Iowa strain of PRRSV. The present polynucleic acid has utility (or usefulness) in the
10 production of the present vaccine, in screening or identifying infected or exposed animals, in identifying related viruses and/or infectious agents, and as a vector for transforming cells and/or immunizing animals (e.g., pigs) with heterologous genes.

15 In the Experiments described hereinbelow, the isolation, cloning and sequencing of ORF's 2-7 of plaque-purified PRRSV isolate ISU-12 (deposited on October 30, 1992, in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A., under
20 the accession numbers VR 2385 [3 x plaque-purified] and VR 2386 [non-plaque-purified]) and ORF's 6-7 of PRRSV isolates ISU-22, ISU-55 and ISU-3927 (deposited on September 29, 1993, in the American Type Culture Collection under the accession numbers VR 2429, VR 2430 and VR 2431,
25 respectively), ISU-79 and ISU-1894 (deposited on August 31, 1994, in the American Type Culture Collection under the

accession numbers _____ and _____, respectively) are described in detail. However, the techniques used to isolate, clone and sequence these genes can be also applied to the isolation, cloning and sequencing of the genomic polynucleic acids of any PRRSV. Thus, the present invention is not limited to the specific sequences disclosed in the Experiments below.

For example, primers for making relatively large amounts of DNA by the polymerase chain reaction (and if desired, for making RNA by transcription and/or protein by translation in accordance with known *in vivo* or *in vitro* methods) can be designed on the basis of sequence information where more than one sequence obtained from a PRRSV genome has been determined (e.g., ORF's 2-5 of VR 2385 and Lelystad virus, or ORF's 6-7 of VR 2385, VR 2429, VR 2430, ISU-79, ISU-1894, VR 2431 and Lelystad virus). A region from about 15 to 50 nucleotides in length having at least 80% and preferably at least 90% identity is selected from the determined sequences. A region where a deletion occurs in one of the sequences (e.g., of at least 5 nucleotides) can be used as the basis for preparing a selective primer for selective amplification of the polynucleic acid of one strain or type of PRRSV over another (e.g., for the differential diagnosis of North American and European PRRSV strains).

Once the genomic polynucleic acid is amplified and cloned into a suitable host by known methods, the clones can be screened with a probe designed on the basis of the sequence information disclosed herein. For example, a
5 region of from about 50 to about 500 nucleotides in length is selected on the basis of either a high degree of identity (e.g., at least 90%) among two or more sequences (e.g., in ORF's 6-7 of the Iowa strains of PRRSV disclosed in Experiment III below), and a polynucleotide of suitable
10 length and sequence identity can be prepared by known methods (such as automated synthesis, or restriction of a suitable fragment from a polynucleic acid containing the selected region, PCR amplification using primers which hybridize specifically to the polynucleotide, and isolation
15 by electrophoresis). The polynucleotide may be labeled with, for example, ³²P (for radiometric identification) or biotin (for detection by fluorometry). The probe is then hybridized with the polynucleic acids of the clones and detected according to known methods.

20 The present Inventors have discovered that ORF 4 appears to be related to the virulence of PRRSV. For example, at least one isolate of PRRSV which shows relatively low virulence also appears to have a deletion in ORF 4 (see, for example, Experiments VIII-XI below).
25 Accordingly, in a preferred embodiment, the present invention is concerned with a polynucleic acid obtained

from a PRRSV isolate which confers immunogenic protection directly or indirectly against a subsequent challenge with a PRRSV, but in which ORF 4 is deleted or mutated to an extent which would render a PRRSV containing the

5 polynucleic acid either low-virulent (i.e., a "low virulence" (lv) phenotype; see the explanation below) or non-virulent (a so-called "deletion mutant"). Preferably, ORF 4 is deleted or mutated to an extent which would render a PRRS virus non-virulent. However, it may be desirable to

10 retain regions of a PRRSV ORF 4 in the present polynucleic acid which (i) encode an antigenic, immunoprotective peptide fragment and (ii) would not confer virulence to a PRRS virus containing the polynucleic acid.

The present invention also encompasses a PRRSV *per se*

15 in which ORF 4 is deleted or mutated to an extent which renders it either low-virulent or non-virulent (e.g., VR 2431). Such a virus is useful as a vaccine or as a vector for transforming a suitable host (e.g., MA-104, PSP 36, CRL 11171, MARC-145 or porcine alveolar macrophage cells) with

20 a heterologous gene. Preferred heterologous genes which may be expressed using the present deletion mutant may include those encoding a protein or an antigen other than a porcine reproductive and respiratory syndrome virus antigen (e.g., pseudorabies and/or swine influenza virus proteins

25 and/or polypeptide-containing antigens, a porcine growth

hormone, etc.) or a polypeptide-based adjuvant (such as those discussed below for the present vaccine composition).

It may also be desirable in certain embodiments of the present polynucleic acid which contain, for example, the
5 3'-terminal region of ORF 3 (e.g., from 200 to 700 nucleotides in length), at least part of which may overlap with the 5'-region of ORF 4. Similarly, where the 3'-terminal region of ORF 4 may overlap with the 5'-terminal region of ORF 5, it may be desirable to retain the 5'-
10 region of ORF 4 which overlaps with ORF 5.

The present Inventors have also discovered that ORF 5 in the PRRSV genome appears to be related to replication of the virus in mammalian host cells capable of sustaining a culture while infected with PRRSV. Accordingly, the
15 present invention is also concerned with polynucleic acids obtained from a PRRSV genome in which ORF 5 may be present in multiple copies (a so-called "overproduction mutant"). For example, the present polynucleic acid may contain at least two, and more preferably, from 2 to 10 copies of ORF
20 5 from a high-replication (hr) phenotype PRRSV isolate.

Interestingly, the PRRSV isolate ISU-12 has a surprisingly large number of potential start codons (ATG/AUG sequences) near the 5'-terminus of ORF 5, possibly indicating alternate start sites of this gene (see SEQ ID
25 NO:13). Thus, alternate forms of the protein encoded by ORF 5 of a PRRSV isolate may exist, particularly where

alternate ORF's encode a protein having a molecular weight similar to that determined experimentally (e.g., from about 150 to about 250 amino acids in length). The most likely coding region for ORF 5 of ISU-12 (SEQ ID NO:14) is indicated in Figure 7.

One can prepare deletion and overproduction mutants in accordance with known methods. For example, one can prepare a mutant polynucleic acid which contains a "silent" or degenerate change in the sequence of a region encoding a polypeptide. By selecting and making an appropriate degenerate mutation, one can substitute a polynucleic acid sequence recognized by a known restriction enzyme. For example, if such a silent, degenerate mutation is made at one or two of the 3'-end of ORF 3 and the 5'- and 3'-ends of ORF 4 and ORF 5, one can insert a synthetic polynucleic acid (a so-called "cassette") which may contain multiple copies of ORF 5, multiple copies of a viral envelope protein or an antigenic fragment thereof. The "cassette" may be preceded by a suitable initiation codon (ATG), and may be suitably terminated with a termination codon at the 3'-end (TAA, TAG or TGA).

Of course, an oligonucleotide sequence which does not encode a polypeptide may be inserted, or alternatively, no cassette may be inserted. By doing so, one may provide a so-called deletion mutant.

Thus, in one embodiment of the present invention, the polynucleic acid encodes one or more proteins, or antigenic regions thereof, of a PRRSV. Preferably, the present nucleic acid encodes at least one antigenic region of a
5 PRRSV membrane (envelope) protein. More preferably, the present polynucleic acid contains at least one copy of the ORF-5 gene from a high virulence (hv) phenotype isolate of PRRSV (see the description of "hv phenotype" below) and a sufficiently long fragment, region or sequence of at least
10 one of ORF-2, ORF-3, ORF-4, ORF-5 and/or ORF-6 from the genome of a PRRSV isolate to encode an antigenic region of the corresponding protein(s) and effectively stimulate immunological protection against a subsequent challenge with an hv phenotype PRRSV isolate. Even more preferably,
15 at least one entire envelope protein encoded by ORF-2, ORF-3, ORF-5 and/or ORF-6 of a PRRSV is contained in the present polynucleic acid, and the present polynucleic acid excludes a sufficiently long portion of ORF 4 from an hv PRRSV to render a PRRSV containing the same either low-
20 virulent or non-virulent. Particularly preferably, the present polynucleic acid excludes the entire region of an hv PRRSV ORF 4 which does not overlap with the 3'-end of ORF 3 and the 5'-end of ORF 5.

Most preferably, the polynucleic acid is isolated from
25 the genome of an isolate of the Iowa strain of PRRSV (for example, VR 2385 (3X plaque-purified ISU-12), VR 2386 (non-

plaque-purified ISU-12), VR 2428 (ISU-51), VR 2429 (ISU-22), VR 2430 (ISU-55), VR 2431 (ISU-3927), ISU-79 and/or ISU-1894.

A preferred embodiment of the present invention
5 concerns a purified preparation which may comprise, consist essentially of or consist of a polynucleic acid having a sequence of the formula (I):



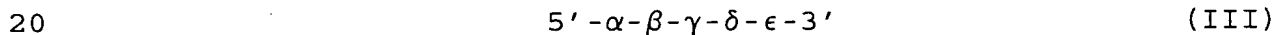
wherein α encodes at least one polypeptide or antigenic
10 fragment thereof encoded by a polynucleotide selected from the group consisting of ORF 2 and ORF 3 of an Iowa strain of PRRSV and regions thereof encoding the antigenic fragments; and β is either a covalent bond or a linking polynucleic acid which excludes a sufficiently long portion
15 of ORF 4 from an hv PRRSV to render the hv PRRSV either low-virulent or non-virulent; and γ is at least one copy of an ORF 5 from an Iowa strain of PRRSV, preferably from a high replication (hr) phenotype.

Alternatively, the present invention may concern a
20 purified preparation which may comprise, consist essentially of or consist of a polynucleic acid having a sequence of the formula (II):



where γ is at least one copy of an ORF 5 from an Iowa strain of PRRSV, preferably from an hv PRRSV isolate; δ is either a covalent bond or a linking polynucleic acid which does not materially affect transcription and/or translation of the polynucleic acid; and ϵ encodes at least one polypeptide or antigenic fragment thereof encoded by a polynucleotide selected from the group consisting of ORF 6 and ORF 7 of an Iowa strain of PRRSV and regions thereof encoding the antigenic fragments; and when δ is a covalent bond, γ may have a 3'-end which excludes the region overlapping with the 5'-end of a corresponding ORF 6. Preferably, ϵ is a polynucleotide encoding at least an antigenic region of a protein encoded by an ORF 6 of an Iowa strain of PRRSV, and more preferably, encodes at least a protein encoded by an ORF 6 of an Iowa strain of PRRSV.

The present invention may also concern a purified preparation which may comprise, consist essentially of or consist of a polynucleic acid having a sequence of the formula (III):



where α , β , γ , δ and ϵ are as defined in formulas (I) and (II) above. Thus, the present polynucleic acid may be selected from the group consisting of, from 5' to 3':

$(\text{ORF } 5)_n$	(IV)
$\zeta - (\text{ORF } 5)_n$	(V)
$(\text{ORF } 5)_{n-\eta}$	(VI)
$\zeta - (\text{ORF } 5)_{n-\eta}$	(VII)

5 where:

ζ is selected from the group consisting of ORF 2-, ORF 3-, ORF 4*-, ORF 2-ORF 3-, ORF 2-ORF 4*-, ORF 3-ORF 4*- and ORF 2-ORF 3-ORF 4*-; and

η is selected from the group consisting of -ORF 5*, -
10 ORF 6, -ORF 7, -ORF 5*-ORF 6, -ORF 5*-ORF 7, -ORF 6-ORF 7
 and -ORF 5*-ORF 6-ORF 7;

 wherein ORF 2, ORF 3, ORF 6 and ORF 7 each encode a
 protein encoded by the second, third, sixth and seventh
 open reading frames of an Iowa strain of PRRSV,
15 respectively; ORF 4* is a region of a fourth open reading
 frame of an Iowa strain of PRRSV which (i) encodes an
 antigenic, immunoprotective peptide fragment and which (ii)
 does not confer virulence to a PRRSV containing the
 polynucleic acid; ORF 5 is a fifth open reading frame of an
20 hv PRRSV isolate; ORF 5* is a region of a fifth open reading
 frame of an Iowa strain of PRRSV which (i) encodes an
 antigenic, immunoprotective peptide fragment and (ii) does
 not confer virulence to a PRRSV containing the polynucleic
 acid, and which may have a 3'-end which excludes the

portion overlapping with the 5'-end of a corresponding ORF 6; and $n \geq 1$.

The present polynucleic acid may also comprise, consist essentially of or consist of combinations of the above sequences, either as a mixture of polynucleotides or covalently linked in either a head-to-tail (sense-antisense) or head-to-head fashion. Polynucleic acids complementary to the above sequences and combinations thereof (antisense polynucleic acid) are also encompassed by the present invention. Thus, in addition to possessing multiple or variant copies of ORF 5, the present polynucleic acid may also contain multiple or variant copies of one or more of ORF's 1-3 and 6-7 and regions of ORF's 4-5 of Iowa strain PRRSV's.

The present invention may also concern polynucleic acids comprising, consisting essentially of or consisting of the open reading frame 1a and 1b from a PRRSV isolate. Based on information regarding viruses evolutionally related to PRRSV, ORF 1a and 1b of PRRSV are believed to encode an RNA polymerase. ORF 1a and 1b are translated into a single protein by frameshifting. Preferably, the polynucleic acid from ORF 1a and 1b of a PRRSV isolate is obtained from an Iowa strain of PRRSV.

Similar to the methods described above and in the following Experiments for ORF's 2-7, one can prepare a library of recombinant clones (e.g., using *E. coli* as a

host) containing suitably prepared restriction fragments of a PRRSV genome (e.g., inserted into an appropriate plasmid expressible in the host). The clones are then screened with a suitable probe (e.g, based on a conserved sequence of ORF's 2-3; see, for example, Figure 22). Positive clones can then be selected and grown to an appropriate level. The polynucleic acids can then be isolated from the positive clones in accordance with known methods. A suitable primer for PCR can then be designed and prepared as described above to amplify the desired region of the polynucleic acid. The amplified polynucleic acid can then be isolated and sequenced by known methods.

The present purified preparation may also contain a polynucleic acid selected from the group consisting of sequences having at least 97% sequence identity (or homology) with at least one ORF 7 of VR 2385, VR 2430 and/or VR 2431; and sequences having at least 80% and preferably at least 90% sequence identity (or homology) with at least one of ORF's 1-6 of VR 2385, VR 2428, VR 2429, VR 2430 and/or VR 2431. Preferably, the polynucleic acid excludes a sufficiently long region or portion of ORF 4 of the hv PRRSV isolates VR 2385, VR 2429, ISU-28, ISU-79 and/or ISU-984 to render the isolate low-virulent or non-virulent.

In the context of the present application, "homology" refers to the percentage of identical nucleotide or amino

acid residues in the sequences of two or more viruses,
aligned in accordance with a conventional method for
determining homology (e.g., the MACVECTOR or GENEWORKS
computer programs, aligned in accordance with the procedure
5 described in Experiment III below).

Accordingly, a further aspect of the present invention
encompasses an isolated polynucleic acid at least 90%
homologous to a polynucleotide which encodes a protein,
polypeptide or fragment thereof encoded by ORF's 1-7 from
10 an Iowa strain of PRRSV (e.g., SEQ ID NOS:15, 17, 19, 43,
45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65 and 67).
Preferably, the present isolated polynucleic acid encodes a
protein, polypeptide, or antigenic fragment thereof which
is at least 10 amino acids in length and in which amino
15 acids non-essential for antigenicity may be conservatively
substituted. An amino acid residue in a protein,
polypeptide, or antigenic fragment thereof is
conservatively substituted if it is replaced with a member
of its polarity group as defined below:

20 Basic amino acids:

lysine (Lys), arginine (Arg), histidine (His)

Acidic amino acids:

aspartic acid (Asp), glutamic acid (Glu),
asparagine (Asn), glutamine (Gln)

Hydrophilic, nonionic amino acids:

serine (Ser), threonine (Thr), cysteine (Cys),
asparagine (Asn), glutamine (Gln)

Sulfur-containing amino acids:

5 cysteine (Cys), methionine (Met)

Hydrophobic, aromatic amino acids:

phenylalanine (Phe), tyrosine (Tyr), tryptophan
(Trp)

Hydrophobic, nonaromatic amino acids:

10 glycine (Gly), alanine (Ala), valine (Val),
leucine (Leu), isoleucine (Ile), proline (Pro)

More particularly, the present polynucleic acid
encodes one or more of the protein(s) encoded by the
second, third, fourth, fifth, sixth and/or seventh open
15 reading frames (ORF's 2-7) of the PRRSV isolates VR 2385,
VR 2386, VR 2428, VR 2429, VR 2430, VR 2431, VR 2432, ISU-
79 and/or ISU-1894 (e.g., SEQ ID NOS:15, 17, 19, 43, 45,
47, 49, 51, 53, 55, 57, 59, 61, 63 and 65).

Relatively short segments of polynucleic acid (about
20 20 bp or longer) in the genome of a virus can be used to
screen or identify tissue and/or biological fluid samples
from infected animals, and/or to identify related viruses,
by methods described herein and known to those of ordinary
skill in the fields of veterinary and viral diagnostics and
25 veterinary medicine. Accordingly, a further aspect of the

present invention encompasses an isolated (and if desired, purified) polynucleic acid consisting essentially of a fragment of from 15 to 2000 bp, preferably from 18 to 1000 bp, and more preferably from 21 to 100 bp in length, derived from ORF's 2-7 of a PRRSV genome (preferably the Iowa strain of PRRSV). Particularly preferably, the present isolated polynucleic acid fragments are obtained from a terminus of one or more of ORF's 2-7 of the genome of the Iowa strain of PRRSV, and most preferably, are selected from the group consisting of SEQ ID NOS:1-12, 22 and 28-34.

The present invention also concerns a diagnostic kit for assaying a porcine reproductive and respiratory syndrome virus, comprising (a) a first primer comprising a polynucleotide having a sequence of from 10 to 50 nucleotides in length which hybridizes to a genomic polynucleic acid from an Iowa strain of porcine reproductive and respiratory syndrome virus at a temperature of from 25 to 75°C, (b) a second primer comprising a polynucleotide having a sequence of from 10 to 50 nucleotides in length, said sequence of said second primer being found in said genomic polynucleic acid from said Iowa strain of porcine reproductive and respiratory syndrome virus and being downstream from the sequence to which the first primer hybridizes, and (c) a reagent which enables detection of an amplified polynucleic acid.

Preferably, the reagent is an intercalating dye, the fluorescent properties of which change upon intercalation into double-stranded DNA.

ORF's 6 and 7 are not likely candidates for
5 controlling virulence and replication phenotypes of PRRSV,
as the nucleotide sequences of these genes are highly
conserved among high virulence (hv) and low virulence (lv)
isolates (see Experiment III below). However, ORF 5 in
PRRSV isolates appears to be less conserved among high
10 replication (hr) and low replication (lr) isolates.
Therefore, it is believed that the presence of an ORF 5
from an hr PRRSV isolate in the present polynucleic acid
will enhance the production and expression of a recombinant
vaccine produced from the polynucleic acid.

15 Accordingly, it is preferred that the present
polynucleic acid, when used for immunoprotective purposes
(e.g., in the preparation of a vaccine), contain at least
one copy of ORF 5 from a high-replication isolate (i.e., an
isolate which grows to a titer of 10^6 - 10^7 TCID₅₀ in, for
20 example, CRL 11171 cells; also see the discussions in
Experiments VIII-XI below).

On the other hand, the lv isolate VR 2431 appears to
be a deletion mutant, relative to hv isolates (see
Experiments III and VIII-XI below). The deletion appears
25 to be in ORF 4, based on Northern blot analysis.
Accordingly, when used for immunoprotective purposes, the

present polynucleic acid preferably does not contain a region of ORF 4 from an hv isolate responsible for its high virulence, and more preferably, excludes the region of ORF 4 which does not overlap with the adjacent ORF's 3 and 5 (where ORF 4 overlaps with the adjacent ORF's 3 and 5).

It is also known (at least for PRRSV) that neither the nucleocapsid protein nor antibodies thereto confer immunological protection against the virus (e.g., PRRSV) to pigs. Accordingly, the present polynucleic acid, when used for immunoprotective purposes, contains one or more copies of one or more regions from ORF's 2, 3, 4, 5 and 6 of a PRRSV isolate encoding an antigenic region of the viral envelope protein, but which does not result in the symptoms or histopathological changes associated with PRRS.

Preferably, this region is immunologically cross-reactive with antibodies to envelope proteins of other PRRSV isolates. Similarly, the protein encoded by the present immunoprotective polynucleic acid confers immunological protection to a pig administered a composition comprising the protein, and antibodies to this protein are immunologically cross-reactive with the envelope proteins of other PRRSV isolates. More preferably, the present immunoprotective polynucleic acid encodes the entire envelope protein of a PRRSV isolate or a protein at least 80% homologous thereto and in which non-homologous residues

are conservatively substituted, or a protein at least 90% homologous thereto.

The present isolated polynucleic acid fragments can be obtained by digestion of the cDNA corresponding to
5 (complementary to) the viral polynucleic acids with one or more appropriate restriction enzymes, can be amplified by PCR and cloned, or can be synthesized using a commercially available automated polynucleotide synthesizer.

Another embodiment of the present invention concerns
10 one or more proteins or antigenic fragments thereof from a PRRS virus, preferably from the Iowa strain of PRRSV. As described above, an antigenic fragment of a protein from a PRRS virus (preferably from the Iowa strain of PRRSV) is at
15 least 5 amino acids in length, particularly preferably at least 10 amino acids in length, and provides or stimulates an immunologically protective response in a pig administered a composition containing the antigenic fragment.

Methods of determining the antigenic portion of a
20 protein are known to those of ordinary skill in the art (see the description above). In addition, one may also determine an essential antigenic fragment of a protein by first showing that the full-length protein is antigenic in a host animal (e.g., a pig). If the protein is still
25 antigenic in the presence of an antibody which specifically binds to a particular region or sequence of the protein,

then that region or sequence may be non-essential for immunoprotection. On the other hand, if the protein is no longer antigenic in the presence of an antibody which specifically binds to a particular region or sequence of the protein, then that region or sequence is considered to be essential for antigenicity.

The present invention also concerns a protein or antigenic fragment thereof encoded by one or more of the polynucleic acids defined above, and preferably by one or more of the ORF's of a PRRSV, more preferably of the Iowa strain of PRRSV. The present proteins and antigenic fragments are useful in immunizing pigs against PRRSV, in serological tests for screening pigs for exposure to or infection by PRRSV (particularly the Iowa strain of PRRSV), etc.

For example, the present protein may be selected from the group consisting of the proteins encoded by ORF's 2-7 of VR 2385, ISU-22 (VR 2429), ISU-55 (VR 2430), ISU-1894, ISU-79 and ISU-3927 (VR 2431) (e.g., SEQ ID NOS:15, 17, 19, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 67, 69 and 71); antigenic regions of at least one of the proteins of SEQ ID NOS:15, 17, 19, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 67, 69 and 71 having a length of from 5 amino acids to less than the full length of the polypeptides of SEQ ID NOS:15, 17, 19, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 67, 69 and 71; polypeptides at least 80% homologous with a

protein encoded by one of the ORF's 2-5 of VR 2385 (SEQ ID NOS:15, 67, 69 and 71); and polypeptides at least 97% homologous with a protein encoded by one of the ORF's 6-7 of VR 2385, VR 2429, VR 2430, ISU-1894, ISU-79 and VR 2431 (e.g., SEQ ID NOS:17, 19, 43, 45, 47, 49, 51, 53, 55, 57, 59 and 61). Preferably, the present protein has a sequence selected from the group consisting of SEQ ID NOS:15, 17, 19, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 67, 69 and 71; variants thereof which provide effective immunological protection to a pig administered the same and in which from 1 to 100 (preferably from 1 to 50 and more preferably from 1 to 25) deletions or conservative substitutions in the amino acid sequence exist; and antigenic fragments thereof at least 5 and preferably at least 10 amino acids in length which provide effective immunological protection to a pig administered the same.

More preferably, the present protein variant or protein fragment has a binding affinity (or association constant) of at least 1% and preferably at least 10% of the binding affinity of the corresponding full-length, naturally-occurring protein to a monoclonal antibody which specifically binds to the full-length, naturally-occurring protein (i.e., the protein encoded by a PRRSV ORF). Most preferably, the present protein has a sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ

ID NO:49, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:55, SEQ ID NO:57, SEQ ID NO:59, SEQ ID NO:61, SEQ ID NO:67, SEQ ID NO:69 and SEQ ID NO:71.

The present invention may also concern a biologically pure virus, characterized in that it contains the present polynucleic acid and/or that it causes a porcine reproductive and respiratory disease which may include one or more of the following histological lesions: gross and/or microscopic lung lesions (e.g., lung consolidation), Type II pneumocytes, myocarditis, encephalitis, alveolar exudate formation and syncytia formation. The phrase "biologically pure" refers to a sample of a virus or infectious agent in which all progeny are derived from a single parent. Usually, a "biologically pure" virus sample is achieved by 3 x plaque purification in cell culture.

In particular, the present biologically pure virus or infectious agent is an isolate of the Iowa strain of porcine reproductive and respiratory syndrome virus, samples of which have been deposited under the terms of the Budapest Treaty at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A., under the accession numbers VR 2385, VR 2386, VR 2428, VR 2429, VR 2430, VR 2431, _____ and _____.

In addition to the characteristics (a)-(e) described above, the Iowa strain of PRRSV may also be characterized by Northern blots of its mRNA. For example, the Iowa

strain of PRRSV may contain either 7 or 9 mRNA's, and may also have deletions or variations in their size. In particular, as will be described in the Experiments below, the mRNA's of the Iowa strain of PRRSV may contain up to
5 four deletions, relative to VR 2385/VR 2386.

The present invention further concerns a composition for protecting a pig from viral infection, comprising an amount of the present vaccine effective to raise an immunological response to a virus which causes a porcine
10 reproductive and respiratory disease in a physiologically acceptable carrier.

An effective amount of the present vaccine is one in which a sufficient immunological response to the vaccine is raised to protect a pig exposed to a virus which causes a
15 porcine reproductive and respiratory disease or related illness. Preferably, the pig is protected to an extent in which from one to all of the adverse physiological symptoms or effects (e.g., lung lesions) of the disease to be prevented are found to be significantly reduced.

20 The composition can be administered in a single dose, or in repeated doses. Dosages may contain, for example, from 1 to 1,000 micrograms of virus-based antigen (vaccine), but should not contain an amount of virus-based antigen sufficient to result in an adverse reaction or
25 physiological symptoms of infection. Methods are known in

the art for determining suitable dosages of active antigenic agent.

The composition containing the present vaccine may be administered in conjunction with an adjuvant or with an acceptable carrier which may prolong or sustain the immunological response in the host animal. An adjuvant is a substance that increases the immunological response to the present vaccine when combined therewith. The adjuvant may be administered at the same time and at the same site as the vaccine or at a different time, for example, as a booster. Adjuvants also may advantageously be administered to the animal in a manner or at a site or location different from the manner, site or location in which the vaccine is administered. Adjuvants include aluminum hydroxide, aluminum potassium sulfate, heat-labile or heat-stable enterotoxin isolated from *Escherichia coli*, cholera toxin or the B subunit thereof, diphtheria toxin, tetanus toxin, pertussis toxin, Freund's incomplete adjuvant, Freund's complete adjuvant, and the like. Toxin-based adjuvants, such as diphtheria toxin, tetanus toxin and pertussis toxin, may be inactivated prior to use, for example, by treatment with formaldehyde.

The present invention also concerns a method of protecting a pig from infection against a virus which causes a porcine reproductive and respiratory disease, comprising administering an effective amount of a vaccine

which raises an immunological response against such a virus to a pig in need of protection against infection by such a virus. By "protecting a pig from infection" against a porcine reproductive and respiratory syndrome virus or
5 infectious agent, it is meant that after administration of the present vaccine to a pig, the pig shows reduced (less severe) or no clinical symptoms (such as fever) associated with the corresponding disease, relative to control (infected) pigs. The clinical symptoms may be quantified
10 (e.g., fever, antibody count, and/or lung lesions), semi-quantified (e.g., severity of respiratory distress), or qualified.

The present invention concerns a system for measuring respiratory distress in affected pigs. The present
15 clinical respiratory scoring system evaluates the respiratory distress of affected pigs by the following scale:

- 0 = no disease; normal breathing
- 20 1 = mild dyspnea and polypnea when the pigs are stressed (forced to breathe in larger volumes and/or at an accelerated rate)
- 2 = mild dyspnea and polypnea when the pigs are at rest
- 25 3 = moderate dyspnea and polypnea when the pigs are stressed
- 4 = moderate dyspnea and polypnea when the pigs are at rest
- 5 = severe dyspnea and polypnea when the pigs are stressed

6 = severe dyspnea and polypnea when the pigs are at rest

In the present clinical respiratory scoring system, a score of "0" is normal, and indicates that the pig is unaffected by a porcine reproductive and respiratory disease. A score of "3" indicates moderate respiratory disease, and a score of "6" indicates very severe respiratory disease. An amount of the present vaccine or composition may be considered effective if a group of challenged pigs given the vaccine or composition show a lower average clinical respiratory score than a group of identically challenged pigs not given the vaccine or composition. (A pig is considered "challenged" when exposed to a concentration of an infectious agent sufficient to cause disease in a non-vaccinated animal.)

Preferably, the present vaccine composition is administered directly to a pig not yet exposed to a virus which causes a reproductive or respiratory disease. The present vaccine may be administered orally or parenterally. Examples of parenteral routes of administration include intradermal, intramuscular, intravenous, intraperitoneal, subcutaneous and intranasal routes of administration.

When administered as a solution, the present vaccine may be prepared in the form of an aqueous solution, a syrup, an elixir, or a tincture. Such formulations are known in the art, and are prepared by dissolution of the

antigen and other appropriate additives in the appropriate solvent systems. Such solvents include water, saline, ethanol, ethylene glycol, glycerol, A1 fluid, etc.

Suitable additives known in the art include certified dyes, 5 flavors, sweeteners, and antimicrobial preservatives, such as thimerosal (sodium ethylmercurithiosalicylate). Such solutions may be stabilized, for example, by addition of partially hydrolyzed gelatin, sorbitol, or cell culture medium, and may be buffered by methods known in the art, 10 using reagents known in the art, such as sodium hydrogen phosphate, sodium dihydrogen phosphate, potassium hydrogen phosphate and/or potassium dihydrogen phosphate.

Liquid formulations may also include suspensions and emulsions. The preparation of suspensions, for example 15 using a colloid mill, and emulsions, for example using a homogenizer, is known in the art.

Parenteral dosage forms, designed for injection into body fluid systems, require proper isotonicity and pH buffering to the corresponding levels of porcine body 20 fluids. Parenteral formulations must also be sterilized prior to use.

Isotonicity can be adjusted with sodium chloride and other salts as needed. Other solvents, such as ethanol or propylene glycol, can be used to increase solubility of 25 ingredients of the composition and stability of the solution. Further additives which can be used in the

present formulation include dextrose, conventional antioxidants and conventional chelating agents, such as ethylenediamine tetraacetic acid (EDTA).

5 The present invention also concerns a method of producing the present vaccine, comprising the steps of synthesizing or isolating a polynucleic acid of a PRRS virus (preferably the Iowa strain) encoding an antigenic protein or portion thereof (preferably the viral coat protein), infecting a suitable host cell with the
10 polynucleic acid, culturing the host cell, and isolating the antigenic protein or portion thereof from the culture. Alternatively, the polynucleic acid itself can confer immunoprotective activity to a host animal to which it is administered.

15 Preferably, the vaccine is collected from a culture medium by the steps of (i) precipitating transfected, cultured host cells, (ii) lysing the precipitated cells, and (iii) isolating the vaccine. Particularly preferably, the host cells infected with the virus or infectious agent
20 are cultured in a suitable medium prior to collecting.

Preferably, after culturing infected host cells, the infected host cells are precipitated by adding a solution of a conventional poly(ethylene glycol) (PEG) to the culture medium, in an amount sufficient to precipitate the
25 infected cells. The precipitated infected cells may be further purified by centrifugation. The precipitated cells

are then lysed by methods known to those of ordinary skill in the art. Preferably, the cells are lysed by repeated freezing and thawing (three cycles of freezing and thawing is particularly preferred). Lysing the precipitated cells
5 releases the virus, which may then be collected, preferably by centrifugation. The virus may be isolated and purified by centrifuging in a CsCl gradient, then recovering the appropriate virus-containing band from the CsCl gradient.

Alternatively, the infected cell culture may be frozen
10 and thawed to lyse the cells. The frozen and thawed cell culture material may be used directly as a live vaccine. Preferably, however, the frozen and thawed cell culture material is lyophilized (for storage), then rehydrated for use as a vaccine.

15 The culture media may contain buffered saline, essential nutrients and suitable sources of carbon and nitrogen recognized in the art, in concentrations sufficient to permit growth of virus-infected cells. Suitable culture media include Dulbecco's minimal essential
20 medium (DMEM), Eagle's minimal essential medium (MEM), Ham's medium, medium 199, fetal bovine serum, fetal calf serum, and other equivalent media which support the growth of virus-infected cells. The culture medium may be supplemented with fetal bovine serum (up to 10%) and/or L-
25 glutamine (up to 2 mM), or other appropriate additives,

such as conventional growth supplements and/or antibiotics.
A preferred medium is DMEM.

Preferably, the present vaccine is prepared from a virus or infectious agent cultured in an appropriate cell
5 line. The cell line is preferably PSP-36 or an equivalent cell line capable of being infected with the virus and cultured. An example of a cell line equivalent to PSP-36 is the cell line PSP-36-SAH, which was deposited under the terms of the Budapest Treaty at the American Type Culture
10 Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A., on October 28, 1992, under the deposit number CRL 11171. Another equivalent cell line is MA-104, available commercially from Whittaker Bioproducts, Inc. (Walkersville, Maryland). Preliminary results indicate
15 that the Iowa strain of PRRSV can also be cultured in porcine turbinate cells.

There also appears to be a relationship between the severity of histopathology caused by a challenge with a standard amount of a particular isolate and the titer to
20 which the isolate can be grown in a mammalian host cell (e.g., CRL 11171, MA-104 cells [from African green monkey kidney], etc.).

Accordingly, the present invention also concerns a method of culturing a PRRS virus, comprising infecting cell
25 line PSP-36, CRL 11171 or an equivalent cell line and culturing the infected cell line in a suitable medium. An

"equivalent cell line" to PSP-36 or CRL 11171 is one which is capable of being infected with the virus and cultured, thereby producing culturable infected cells. Equivalent cell lines include MA-104, PSP-36-SAH and MARC-145 cells
5 (available from the National Veterinary Services Laboratory, Ames, Iowa), for example.

Preferably, the virus cultured is at least one isolate of the Iowa strain of PRRSV. Particularly preferably, the present vaccine is prepared from such a culture of the Iowa
10 strain of PRRSV, cultivated in PSP-36 cells, and plaque-purified at least three times.

The cell line MA-104 is obtained from monkey kidney cells, and is epithelial-like. MA-104 cells form a confluent monolayer in culture flasks containing Dulbecco's
15 minimal essential medium and 10% FBS (fetal bovine serum). When the monolayer is formed, the cells are inoculated with a sample of 10% homogenized tissue, taken from an appropriate tissue (such as lung and/or heart) in an infected pig. Preferably, appropriate antibiotics are
20 present, to permit growth of virus and host cells and to suppress growth and/or viability of cells other than the host cells (e.g., bacteria or yeast).

Both PSP-36 and MA-104 cells grow some isolates of the PRRS virus to high titers (over 10^7 TCID₅₀/ml). PSP-36 and
25 MA-104 cells will also grow the infectious agent associated

with the Iowa strain of PRRSV. MA-104 cells also are able to grow rotaviruses, polioviruses, and other viruses.

CL2621 cells are believed to be of non-porcine origin and are epithelial-like, and are proprietary (Boehringer-
5 Ingelheim). By contrast to PSP-36 and MA-104, some samples of the virus which causes PRRS have been unsuccessfully cultured in CL2621 cells (Bautista et al, *American Association of Swine Practitioners Newsletter*, 4:32, 1992).

The primary characteristics of CL2621 are that it is
10 of non-swine origin, and is epithelial-like, growing in MEM medium. However, Benfield et al (*J. Vet. Diagn. Invest.*, 1992; 4:127-133) have reported that CL2621 cells were used to propagate PRRS virus, but MA-104 cells were used to control polio virus propagation, thus inferring that CL2621
15 is not the same as MA-104, and that the same cell may not propagate both viruses.

The Iowa strain of PRRSV generally cannot grow in cell lines other than PSP-36, PSP-36-SAH and MA-104. As described above, however, some viruses which cause PRRS
20 have been reported to grow in both CL2621 and primary swine alveolar macrophages, although some strains of PRRS virus do not grow in PSP-36, MA-104 or CL2621 cells.

The present vaccine, virus isolates, proteins and polynucleic acids can be used to prepare antibodies which
25 may provide immunological resistance to a patient (in this case, a pig) exposed to a virus or infectious agent.

Antibodies encompassed by the present invention immunologically bind either to (1) a vaccine which protects a pig against a PRRS virus or (2) to the PRRS virus itself. The present antibodies also have the following utilities:

5 (1) as a diagnostic agent for determining whether a pig has been exposed to a PRRS virus or infectious agent, and (2) in the preparation of the present vaccine. The present antibody may be used to prepare an immunoaffinity column by known methods, and the immunoaffinity column can be used to
10 isolate the virus or infectious agent, or a protein thereof.

To raise antibodies to such vaccines or viruses, one immunizes an appropriate host animal, such as a mouse, rabbit, or other animals used for such inoculation, with
15 the protein used to prepare the vaccine. The host animal is then immunized (injected) with one of the types of vaccines described above, optionally administering an immune-enhancing agent (adjuvant), such as those described above. The host animal is preferably subsequently
20 immunized from 1 to 5 times at certain intervals of time, preferably every 1 to 4 weeks, most preferably every 2 weeks. The host animals are then sacrificed, and their blood is collected. Sera is then separated by known techniques from the whole blood collected. The sera
25 contains antibodies to the vaccines. Antibodies can also

be purified by known methods to provide immunoglobulin G (IgG) antibodies.

The present invention also encompasses monoclonal antibodies to the present vaccines and/or viruses.

5 Monoclonal antibodies may be produced by the method of Kohler et al (*Nature*, vol. 256 (1975), pages 495-497).

Basically, the immune cells from a whole cell preparation of the spleen of the immunized host animal (described above) are fused with myeloma cells by a conventional

10 procedure to produce hybridomas. Hybridomas are cultured, and the resulting culture fluid is screened against the fluid or inoculum carrying the infectious agent (virus or vaccine). Introducing the hybridoma into the peritoneum of the host animal produces a peritoneal growth of the

15 hybridoma. Collection of the ascites fluid of the host animal provides a sample of the monoclonal antibody to the infectious agent produced by the hybridoma. Also, supernatant from the hybridoma cell culture can be used as a source of the monoclonal antibody, which is isolated by
20 methods known to those of ordinary skill in the art.

Preferably, the present antibody is of the IgG or IgM type of immunoglobulin.

The present invention also concerns a method of treating a pig suffering from a reproductive and
25 respiratory disease, comprising administering an effective amount of an antibody which immunologically binds to a

virus which causes a porcine reproductive and respiratory disease or to a vaccine which protects a pig against infection by a porcine reproductive and respiratory virus in a physiologically acceptable carrier to a pig in need thereof.

The present method also concerns a method of diagnosing infection of a pig by or exposure of a herd to a porcine reproductive and respiratory syndrome virus and a diagnostic kit for assaying the same, comprising the present antibody (preferably a monoclonal antibody) and a diagnostic agent which indicates a positive immunological reaction with said antibody (preferably comprising peroxidase-conjugated streptavidin, a biotinylated antibody to a PRRSV protein or antigen and a peroxidase). The present kit may further comprise aqueous hydrogen peroxide, a protease which digests the porcine tissue sample, a fluorescent dye (e.g., 3,3'-diaminobenzidine tetrahydrochloride), and a tissue stain (e.g., hematoxylin).

A diagnosis of PRRS relies on compiling information from the clinical history of the herd being diagnosed, from serology and pathology of infected pigs, and ultimately, on isolation of the PRRS virus (PRRSV) from the infected herd. Thus, the present method of detecting PRRSV is useful in diagnosing infection by and/or exposure to the virus in a herd.

Clinical signs vary widely between farms, and thus, are not the most reliable evidence of a definitive diagnosis, except in the case of a severe acute outbreak in naive herds which experience abortion storms, increased numbers of stillborn pigs, and severe neonatal and nursery pig pneumonia. Presently, the most common clinical presentation is pneumonia and miscellaneous bacterial problems in 3-10 week old pigs. However, many PRRSV-positive herds have no apparent reproductive or respiratory problems.

There are some gross lesions that are very suggestive of PRRSV infection in growing pigs. The most consistent experimentally reproducible gross lesion in 3-10 week-old pigs inoculated with several different PRRSV strains is lymphadenopathy. In particular, iliac and mediastinal lymph nodes are often 3-10 times normal size, tan in color, and sometimes cystic. The lymph nodes are not normally hyperemic, such as the lesion/conditions seen in bacterial septicemia.

Three histologic lesions are consistent with PRRSV infection. Interstitial pneumonia is commonly observed and is characterized by septal infiltration with mononuclear cells, type 2 pneumocyte proliferation, and the presence of necrotic cells in the alveolar spaces. Nonsuppurative perivascular myocarditis and hyperplastic lymph nodes are commonly observed in the subacute stages of disease.

The degree of grossly visible pneumonia is strain dependent. In general, the lungs fail to collapse and have a patchy distribution of 10-80% tan-colored consolidation with irregular borders. Encephalitis is less often
5 observed. Lesions in the fetus and placenta are rarely observed by light microscopy.

However, the percentage of consolidation in the lungs provides a particularly reliable test for infection by PRRSV (i.e., $\geq 10\%$ consolidation at any time from 3 to 10
10 days post-infection (DPI) is a positive indication of infection), particularly by a high virulence phenotype (hv) virus ($\geq 40\%$ consolidation at any time from 3 to 10 days DPI is a positive indication of infection by an hv PRRSV isolate).

15 In contrast to histopathology on lung tissue(s), most laboratories are routinely using either an indirect-fluorescent antibody (IFA) test or immunoperoxidase monolayer assay (IPMA) for serum antibody detection. With both the IFA and IPMA, one must subjectively determine
20 endpoints and thus the tests are not automatable. Serum virus (SVN) neutralization tests have also been developed, and ELISA tests are currently used in some research laboratories. Antibodies detected by the IFA test usually appear with 10 days of exposure but may be relatively
25 short-lived, sometimes disappearing within 3 months.

Antibodies detected by ELISA usually appear within 3 weeks, but their duration is unknown. SVN antibodies usually are not detected until 4-5 weeks post exposure. The SVN test is considered less sensitive in acute disease, but improvements have been made in the SVN test using seronegative porcine serum supplementation. SVN titers reportedly are measurable longer than titers in IFA and IPMA, and thus, may be better suited for detection of positive animals in chronically infected herds.

10 In IFA, infected cells are fixed with acetone and methanol solutions, and antibodies for the convalescent sera of infected pigs are incubated with the infected cells, preferably for about 30 min. at 37°C. A positive immunological reaction is one in which the antibody binds to the virus-infected cells, but is not washed out by subsequent washing steps (usually 3 X with PBS buffer). A second antibody (an anti-antibody) labeled with a fluorescent reagent (FITC) is then added and incubated, preferably for another 30 min. A positive immunological reaction results in the second antibody binding to the first, being retained after washing, and resulting in a fluorescent signal, which can be detected and semi-quantified. A negative immunological reaction results in little or no binding of the antibody to the infected cell. Therefore, the second, fluorescently-labeled antibody fails to bind, the fluorescent label is washed out, and little or

no fluorescence is detected, compared to an appropriate positive control.

IPA and ELISA kits are similar to the IFA kit, except that the second antibody is labeled with a specific enzyme, instead of a fluorescent reagent. Thus, one adds an appropriate substrate for the enzyme bound to the second antibody which results in the production of a colored product, which is then detected and quantified by colorimetry, for example.

10 Clinicians use antibody titers to determine the appropriate time for vaccination and/or implementation of management or control strategies. Prior to the present invention, serology tests did not provide antibody titer levels adequate or reliable enough to make animal health care decisions. It may have been appropriate to look for a change from seronegative to seropositive status, or for at least a 4-fold increase in titer, as a positive indication of PRRSV infection/exposure. Looking for an increasing percentage of seropositive pigs in a particular age group over time in a herd can be useful to determine where the virus is maintained and actively spreading. Sows infected in the early 3rd trimester and aborting near term will likely not show increasing titers, however.

25 Virus isolation (VI) provides a definitive diagnosis, but has some limitations. Virus is rarely isolated from stillborn or autolyzed aborted fetuses. Sows infected

early in the last trimester may have transient viremia and not abort until late term. Dead pigs of any age are not the best samples for VI, because the virus does not survive well at room temperature. Tissues should be removed from the carcass, packaged separately, and refrigerated as soon as possible to obtain a viable virus sample.

The best tissues for virus isolation are tonsil, lung, lymph nodes, and spleen. Serum is also an excellent sample for virus isolation, since (a) viremia is often prolonged in growing pigs, (b) the sample is easy to handle, and (c) the sample can be quickly chilled and processed.

Variation between laboratories in the ability to isolate PRRSV is high because the tests, reagents, cell lines, and media used to detect/screen for PRRSV have not been standardized. The efficacy of isolation varies because not all North American strains will grow on each cell line. Frozen tissue-section IFA tests have been used with limited success.

Serum virus neutralization (SVN) tests have also been developed, and ELISA tests are currently used in some research laboratories. Antibodies detected by ELISA usually appear within 3 weeks, but their duration is unknown. SVN antibodies usually are not detected until 4-5 weeks post-exposure. The SVN test is considered less sensitive in acute disease, but improvements have been made in the SVN test using seronegative porcine serum

supplementation. SVN titers reportedly are measurable for a longer period of time than titers in IFA and IPMA. Thus, SVN titers may be better suited for detection of positive animals in chronically infected herds.

5 Prior to the present invention, however, serology tests did not provide antibody titer levels adequate or reliable enough to make animal health care decisions. Looking for an increasing percentage of seropositive pigs in a particular age group over time in a herd can also be
10 useful to determine where the virus is maintained and actively spreading. Sows infected in the early third trimester and aborting near term will likely not show increasing titers, however. Thus, although it may have been appropriate to look for a change from seronegative to
15 seropositive status or for at least a 4-fold increase in titer as a positive indication of PRRSV infection and/or exposure, a need for a more reliable titer-based assay is felt.

 Thus, the present invention also concerns a method for
20 detecting PRRSV antigen in tissues. The present diagnostic method, employing an immunoperoxidase test (IPT) preferably on formalin-fixed tissue, appears to be quite useful to confirm the presence of active infection, and may provide a significant and meaningful increase in the reliability of
25 titer-based assays. A section of lungs, tonsils, mediastinal lymph nodes, and tracheobronchial lymph nodes

from 26 pigs experimentally inoculated with ATCC VR 2385 PRRSV was examined (see Experiment V below). The virus was detected in 18/26 lungs, 26/26 tonsils, 15/26 mediastinal lymph nodes, and 14/26 tracheobronchial lymph nodes. The
5 pigs in this study were killed over a 28 day period (post-inoculation). The virus was detected in at least one tissue in every pig necropsied up to 10 days post inoculation.

A complete technique for the present immunoperoxidase
10 technique for PRRSV antigen detection in porcine tissues, based on a streptavidin-biotin assay, is described in Example V hereinunder. Briefly, the present method for detecting PRRSV comprises removing endogenous peroxidase from an isolated porcine tissue sample with aqueous
15 hydrogen peroxide (preferably, a 0.1-5%, and more preferably, 0.1-1.0% solution), then digesting the tissue with sufficient amount of an appropriate protease to expose viral antigens (for example, Protease XIV, Sigma Chemical Company, St. Louis, MO, and more preferably, a 0.001-0.25%
20 aqueous solution thereof). Thereafter, the method further comprises incubating primary monoclonal antibody ascites fluid (preferably diluted in TRIS/PBS by an amount of from 1:10 to 1:100,000, and more preferably, from 1:100 to 1:10,000) with the protease-treated tissue sections in a
25 humidified chamber for a sufficient length of time and at an appropriate temperature to provide essentially complete

immunological binding to occur, if it can in fact occur (e.g., 16 hours at 4°C).

One suitable monoclonal antibody for use in the present diagnostic assay is SDOW-17 (available from Dr. David Benfield, South Dakota State Univ.), which recognizes a conserved epitope of the PRRSV nucleocapsid protein (Nelson et al, "Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies," *J. Clin. Micro.*, 31:3184-3189 (1993)).

The present method for detecting PRRSV then further comprises incubating biotinylated goat anti-mouse linking antibody (available from Dako Corporation, Carpintera, CA) with the tissue, followed by incubating peroxidase-conjugated streptavidin with the biotinylated antibody-treated tissue (Zymed Laboratories, South San Francisco, CA). The method then further comprises incubating the peroxidase-conjugated streptavidin-treated tissue with a chromagen, such as 3,3'-diaminobenzidine tetrahydrochloride (available from Vector Laboratories Inc., Burlingame, CA), and finally, staining the treated tissue with hematoxylin.

Particularly when combined with the further diagnostic techniques of histopathology, virus isolation procedures and serology, the present tissue immunoperoxidase antigen detection technique offers a rapid and reliable diagnosis of PRRSV infection.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments, which are given for illustration of the invention, and are not intended to be limiting thereof.

5

EXPERIMENT I

MOLECULAR CLONING AND NUCLEOTIDE SEQUENCING OF THE 3'-TERMINAL REGION OF VR 2385 (PLAQUE-PURIFIED ISU-12)

(I) Materials and Methods

(A) Virus Propagation and Purification

10 A continuous cell line, PSP-36, was used to isolate and propagate ISU-12. The ISU-12 virus was plaque-purified 3 times on PSP-36 cells (plaque-purified ISU-12 virus was deposited under the terms and conditions of the Budapest Treaty at the American Type Culture Collection, 12301
15 Parklawn Drive, Rockville, Maryland 20852, U.S.A., under Accession No. VR 2385). The PSP-36 cells were then infected with the plaque-purified virus. When more than 70% of the infected cells showed cytopathic changes, the culture was frozen and thawed three times. The culture
20 medium was then clarified by low-speed centrifugation at 5,000 X g for 15 min. at 4°C. The virus was then precipitated with 7% PEG-8000 and 2.3% NaCl at 4°C overnight with stirring, and the precipitate was pelleted by centrifugation. The virus pellets were then resuspended
25 in 2 ml of tris-EDTA buffer, and layered on top of a CsCl

gradient (1.1245-1.2858 g/ml). After ultracentrifugation at 28,000 rpm for about 8 hours at 20°C, a clear band with a density of 1.15-1.18 g/ml was observed and harvested. The infectivity titer of this band was determined by IFA, and the titer was found to be 10^6 TCID₅₀/ml. Typical virus particles were also observed by negative staining electron microscopy (EM).

(B) Isolation of Viral RNA

Total RNA was isolated from the virus-containing band in the CsCl gradient with a commercially available RNA isolation kit (obtained from Stratagene). Poly(A) RNA was then enriched by oligo (dT)-cellulose column chromatography according to the procedure described by the manufacturer of the column (Invitrogen).

(C) Construction of VR 2385 cDNA λ library

A general schematic procedure for the construction of a cDNA λ library is shown in Figure 3. First strand cDNA synthesis from mRNA was conducted by reverse transcription using an oligo (dT) primer having a Xho I restriction site. The nucleotide mixture contained normal dATP, dGTP, dTTP and the analog 5-methyl dCTP, which protects the cDNA from restriction enzymes used in subsequent cloning steps.

Second strand cDNA synthesis was then conducted with RNase H and DNA polymerase I. The cDNA termini were

blunted (blunt-ended) with T4 DNA polymerase, ligated to EcoR I adaptors with T4 DNA ligase, and subsequently phosphorylated with T4 polynucleotide kinase. The cDNA was digested with Xho I, and the digested cDNA were size-
5 selected on an agarose gel. Digested cDNA larger than 1 kb in size were selected and purified by a commercially available DNA purification kit (GENECLEAN, available from BIO 101, Inc., La Jolla, California).

The purified cDNA was then ligated into lambda phage
10 vector arms, engineered with Xho I and EcoR I cohesive ends. The ligated vector was packaged into infectious lambda phages with lambda extracts. The SURE strain (available from Stratagene) of *E. coli* cells were used for transfection, and the lambda library was then amplified and
15 titrated in the XL-1 blue cell strain.

(D) Screening the λ Library by Differential

Hybridization

A general schematic procedure for identifying authentic clones of the PRRS virus VR 2385 strain by
20 differential hybridization is shown in Figure 4, and is described hereunder. The λ library was plated on XL-1 blue cells, plaques were lifted onto nylon membranes in duplicates, and denatured with 0.5 N NaOH by conventional methodology. Messenger RNA's from both virus-infected PSP-
25 36 cells and non-infected PSP-36 cells were isolated by

oligo (dT)cellulose column chromatography as described by the manufacturer of the column (Invitrogen).

Complementary DNA probes were synthesized from mRNA's isolated from virus-infected PSP-36 cells and normal PSP-36 cells using random primers in the presence of ^{32}P -dCTP according to the procedure described by the manufacturer (Amersham). Two probes (the first synthesized from virus-infected PSP-36 cells, the other from normal, uninfected PSP-36 cells) were then purified individually by Sephadex G-50 column chromatography. The probes were hybridized with the duplicated nylon membranes, respectively, at 42°C in 50% formamide. Plaques which hybridized with the probe prepared from virus infected cells, but not with the probe prepared from normal cells, were isolated. The phagemids containing viral cDNA inserts were rescued by *in vitro* excision with the help of G408 helper phage. The rescued phagemids were then amplified on XL-1 blue cells. The plasmids containing viral cDNA inserts were isolated by Qiagen column chromatography, and were subsequently sequenced.

(E) Nucleotide Sequencing and Sequence Analysis

Plasmids containing viral cDNA inserts were purified by Qiagen column chromatography, and sequenced by Sanger's dideoxy method with universal and reverse primers, as well as a variety of internal oligonucleotide primers.

Sequences were obtained from at least three separate clones. Additional clones or regions were sequenced when ambiguous sequence data were obtained. The nucleotide sequence data were assembled and analyzed independently using two computer software programs, GENEWORKS (IntelliGenetics, Inc., Mountain View, California) and MACVECTOR (International Biotechnologies, Inc., New Haven, Connecticut).

(F) Oligonucleotide Primers

Oligonucleotides were synthesized as single-stranded DNA using an automated DNA synthesizer (Applied Biosystems) and purified by HPLC. Oligonucleotides PP284 (5'-CGGCCGTGTG GTTCTCGCCA AT-3'; SEQ ID NO:1) and PP285 (5'-CCCCATTTC CTCTAGCGAC TG-3'; SEQ ID NO:2) were synthesized for PCR amplification. A DNA probe was generated with these two primers from the extreme 3' end of the viral genome for Northern blot analysis (see discussion below). Oligonucleotides PP286 (5'-GCCGCGGAAC CATCAAGCAC-3'; SEQ ID NO:3) and PP287 (5'-CAACTTGACG CTATGTGAGC-3'; SEQ ID NO:4) were synthesized for PCR amplification. A DNA probe generated by these two primers was used to further screen the λ library. Oligonucleotides PP288 (5'-GCGGTCTGGA TTGACGACAG-3'; SEQ ID NO:5), PP289 (5'-GACTGCTAGG GCTTCTGCAC-3'; SEQ ID NO:6), PP386 (5'-GCCATTCAGC

TCACATAGCG-3'; SEQ ID NO:7), PP286 and PP287 were used as sequencing primers to obtain internal sequences.

(G) Northern Blot Analysis

A specific DNA fragment from the extreme 3' end of the
5 VR 2385 cDNA clone was amplified by PCR with primers PP284
and PP285. The DNA fragment was excised from an agarose
gel with a commercially available DNA purification kit
(GENECLEAN, obtained from Bio 101), and labeled with
³²P-dCTP by random primer extension (using a kit available
10 from Amersham). Total RNA was isolated from VR 2385-
infected PSP-36 cells at 36 hours post-infection, using a
commercially available kit for isolation of total RNA
according to the procedure described by the manufacturer
(Stratagene). VR 2385 subgenomic mRNA species were
15 denatured with 6 M glyoxal and DMSO, and separated on a 1%
agarose gel. (Results from a similar procedure
substituting a 1.5% agarose gel are described in Experiment
II below and are shown in Figure 5.) The separated
subgenomic mRNA's were then transferred onto nylon
20 membranes using a POSIBLOT™ pressure blotter (Stratagene).
Hybridization was carried out in a hybridization oven with
roller bottles at 42°C and 50% formamide.

RESULTS

(A) Cloning, Identification and Sequencing of VR 2385 3' Terminal Genome

An oligo (dT)-primed cDNA λ library was constructed
5 from a partially purified virus, obtained from VR 2385-
infected PSP-36 cells. Problems were encountered in
screening the cDNA λ library with probes based on the
Lelystad virus sequence. Three sets of primers were
prepared. The first set (PP105 and PP106; SEQ ID NOS:8-9)
10 correspond to positions 14577 to 14596 and 14977 to 14995
of the Lelystad genomic sequence, located in the
nucleocapsid gene region. The second set (PP106 and PP107,
SEQ ID NOS:9-10) correspond to positions 14977 to 14995 and
14054 to 14072 of the Lelystad genomic sequence, flanking
15 ORF's 6 and 7. The third set (PM541 and PM542; SEQ ID
NOS:11-12) correspond to positions 11718 to 11737 and 11394
to 11413 of the Lelystad genomic sequence, located in the
ORF-1b region.

PP105: 5'-CTCGTCAAGT ATGGCCGGT-3' (SEQ ID NO:8)
20 PP106: 5'-GCCATTCGCC TGACTGTCA-3' (SEQ ID NO:9)
PP107: 5'-TTGACGAGGA CTTCGGCTG-3' (SEQ ID NO:10)
PM541: 5'-GCTCTACCTG CAATTCTGTG-3' (SEQ ID NO:11)
PM542: 5'-GTGTATAGGA CCGGCAACCG-3' (SEQ ID NO:12)

All attempts to generate probes by PCR from the VR
25 2385 infectious agent using these three sets of primers

were unsuccessful. After several attempts using the differential hybridization technique, however, the authentic plaques representing VR 2385-specific cDNA were isolated using probes prepared from VR 2385-infected PSP-36
5 cells and normal PSP-36 cells. The procedures involved in differential hybridization are described and set forth in Figure 4.

Three positive plaques (λ -4, λ -75 and λ -91) were initially identified. Phagemids containing viral cDNA
10 inserts within the λ phage were rescued by *in vitro* excision with the help of G408 helper phages. The inserts of the positive clones were analyzed by restriction enzyme digestion and terminal sequencing. The specificity of the cDNA clones was further confirmed by hybridization with RNA
15 from PSP-36 cells infected with the Iowa strain of PRRSV, but not with RNA from normal PSP-36 cells. A DNA probe was then generated from the 5'-end of clone λ -75 by PCR with primers PP286 and PP287. Further positive plaques (λ -229, λ -268, λ -275, λ -281, λ -323 and λ -345) were identified using
20 this probe. All λ cDNA clones used to obtain the 3'-terminal nucleotide sequences are presented in Fig. 6. At least three separate clones were sequenced to eliminate any mistakes. In the case of any ambiguous sequence data, additional clones and internal primers (PP288, PP289,
25 PP286, PP287 and PP386) were used to determine the sequence. The 2062-bp 3'-terminal sequence (SEQ ID NO:13)

and the amino acid sequences encoded by ORF's 5, 6 and 7 (SEQ ID NOS:15, 17 and 19, respectively) are presented in Figure 7.

(B) A Nested Set of Subgenomic mRNA

5 Total RNA from virus-infected PSP-36 cells was separated on 1% glyoxal/DMSO agarose gel, and blotted onto nylon membranes. A cDNA probe was generated by PCR with a set of primers (PP284 and PP285) flanking the extreme 3'-terminal region of the viral genome. The probe contains a
10 3'-nontranslational sequence and most of the ORF-7 sequence. Northern blot hybridization results show that the pattern of mRNA species from PSP-36 cells infected with the Iowa strain of PRRSV is very similar to that of Lelystad virus (LV), equine arteritis virus (EAV), lactate
15 dehydrogenase-elevating virus (LDV) and coronavirus, in that virus replication required the formation of subgenomic mRNA's.

 The results also indicate that VR 2385-specific subgenomic mRNA's represent a 3'-nested set of mRNA's,
20 because the Northern blot probe represents only the extreme 3' terminal sequence. The size of VR 2385 viral genomic RNA (14 kb) and 6 subgenomic mRNA's (RNA 2 (3.0 kb), RNA 3 (2.5 kb), RNA 4 (2.2 kb), RNA 5 (1.8 kb), RNA 6 (1.3 kb) and RNA 7 (0.98 kb)) resemble those of LV, although there
25 are differences in both the genome and in subgenomic RNA

species. Differences were also observed in the relative amounts of the subgenomic mRNA's, RNA 7 being the most predominant subgenomic mRNA.

(C) Analysis of Open Reading Frames Encoded by
5 Subgenomic RNA

Three large ORF's have been found in SEQ ID NO:13:
ORF-5 (nucleotides [nt] 426-1025; SEQ ID NO:14), ORF 6 (nt
1013-1534; SEQ ID NO:16) and ORF 7 (nt 1527-1895; SEQ ID
NO:18). ORF 4, located at the 5' end of the resulting
10 sequence, is incomplete in the 2062-bp 3'-terminal sequence
of SEQ ID NO:13. ORF'S 5, 6 AND 7 each have a coding
capacity of more than 100 amino acids. ORF 5 and ORF 6
overlap each other by 13 bp, and ORF 6 and ORF 7 overlap
each other by 8 bp. Two smaller ORF's located entirely
15 within ORF 7 have also been found, coding for only 37 aa
and 43 aa, respectively. Another two short ORF's overlap
fully with ORF 5. The coding capacity of these two ORF's
is only 29 aa and 44 aa, respectively. No specific
subgenomic mRNA's were correlated to these smaller ORF's by
20 Northern blot analysis. ORF 6 and ORF 7 are believed to
encode the viral membrane protein and capsid protein,
respectively.

(D) Consensus Sequence for Leader Junction

Sequence analysis shows that a short sequence motif, AACC, may serve as the site in the subgenomic mRNA's where the leader is added during transcription (the junction site). The junction site of ORF 6 is found 21 bp upstream from the ATG start codon, and the junction site of ORF 7 is found 13 bp upstream from the ATG start codon, respectively. No AACC consensus sequence has been identified in ORF 5, although it has been found in ORF 5 of LV. Similar junction sequences have been found in LDV and EAV.

(E) 3'-Nontranslational Sequence and Poly (A) Tail

A 151 nucleotide-long (151 nt) nontranslational sequence following the stop codon of ORF 7 has been identified in the genome of VR 2385, compared to 114 nt in LV, 80 nt in LDV and 59 nt in EAV. The length of the poly (A) tail is at least 13 nucleotides. There is a consensus sequence, CCGG/AAATT-poly (A) among PRRS virus VR 2385, LV and LDV in the region adjacent to the poly (A) tail.

(F) Sequence Comparison of VR 2385 and LV Genomes
Among ORF's 5, 6 and 7, and Among the
Nontranslational Sequences

A comparison of the ORF-5 regions of the genomes of VR 2385 and of the Lelystad virus (SEQ ID NO:20) is shown in

Figure 8. The corresponding comparisons of the ORF-6 region, the ORF-7 region, and the nontranslational sequences of VR 2385 (SEQ ID NOS:16, 18 and 22, respectively) with the corresponding regions of LV (SEQ ID NOS:23, 25 and 27, respectively) are shown in Figures 9, 10 and 11, respectively.

The results of the comparisons are presented in Table 1 below. The nucleotide sequence homologies between LV and VR 2385 of the ORF 5, ORF 6, ORF 7 and the nontranslational sequences are 53%, 78%, 58% and 58%, respectively.

The size of ORF 7 in LV is 15 nt larger than that in VR 2385. Also, the 3'-terminal nontranslational sequence is different in length (150 nt in VR 2385, but only 114 nt in LV). Like LV, the junction sequence, AACC, has also been identified in the genome of the Iowa strain of PRRS virus isolate VR 2385, except for ORF 5. The junction sequence of ORF 6 in VR 2385 is 21 nt upstream from the ATG start codon, whereas the junction sequence of ORF 6 is 28 nt upstream from ATG in LV.

Table 1: Comparison of genes of U.S. PRRSV isolate ATCC VR 2385 with those of European isolate Lelystad virus*

Gene	RNA	Estimated RNA size (in Kb)	ORFs	VR 2385			Lelystad			Homology between VR 2385 & Lelystad
				Size amino acids	N-glycosylation sites	Pred. protein size (kd)	Size amino acids	N-glycosylation sites	Pred. protein size (kd)	
5	5	1.9	5	200	2	22.2	201	2	22.4	53
6	6	1.4	6	174	1	19.1	173	2	18.9	78
7	7	0.9	7	123	2	13.6	128	1	13.8	58
NTR	-	-	-	151 (nt)	-	NA	114 (nt)	0	NA	58 (nt)

*: Based on data presented by Conzelmann et al, *Virology*, 193, 329-339 (1993), Meulenberg et al, *Virology*, 192, 62-72 (1993), and the results presented herein.

EXPERIMENT II

5 THE EXPRESSION OF VR 2385 GENES IN INSECT CELLS

(A) Production of Recombinant Baculovirus

The ORF-5, ORF-6 and ORF-7 sequences were individually amplified by PCR using primers based on the VR 2385 (ISU-12) genomic nucleotide sequence. ORF-5 was amplified using the following primers:

5'-GGGGATCCGG TATTTGGCAA TGTGTC-3' (SEQ ID NO:28)

3'-GGGAATTCGC CAAGAGCACC TTTTGTGG-5' (SEQ ID NO:29)

ORF-6 was amplified using the following primers:

5'-GGGGATCCAG AGTTTCAGCG G-3' (SEQ ID NO:30)

3'-GGGAATTCTG GCACAGCTGA TTGAC-5' (SEQ ID NO:31)

ORF-7 was amplified using the following primers:

5'-GGGGATCCTT GTTAAATATG CC-3' (SEQ ID NO:32)

3'-GGGAATTCAC CACGCATTC-5' (SEQ ID NO:33)

5 The amplified DNA fragments were cloned into
baculovirus transfer vector pVL1393 (available from
Invitrogen). One μ g of linearized baculovirus AcMNPV DNA
(commercially available from Pharmingen, San Diego,
California) and 2 μ g of PCR-amplified cloned cDNA-
10 containing vector constructs were mixed with 50 μ l of
lipofectin (Gibco), and incubated at 22°C for 15 min. to
prepare a transfection mixture.

 One hour after seeding HI-FIVE cells, the medium was
replaced with fresh Excell 400 insect cell culture medium
15 (available from JR Scientific Co.), and the transfection
mixture was added drop by drop. The resulting mixture was
incubated at 28°C for six hours. Afterwards, the
transfection medium was removed, and fresh Excell 400
insect cell culture medium was added. The resulting
20 mixture was then incubated at 28°C.

 Five days after transfection, the culture medium was
collected and clarified. Ten-fold dilutions of
supernatants were inoculated onto HI-FIVE cells, and
incubated for 60 min. at room temperature. After the
25 inoculum was discarded, an overlay of 1.25% of agarose was

applied onto the cells. Incubation at 28°C was conducted for four days. Thereafter, clear plaques were selected and picked using a sterile Pasteur pipette. Each plaque was mixed with 1 ml of Grace's insect medium into a 5 ml snap cap tube, and placed in a refrigerator overnight to release the virus from the agarose. Tubes were centrifuged for 30 minutes at 2000 x g to remove agarose, and the supernatants were transferred into new sterile tubes. Plaque purification steps were repeated three times to avoid possible wild-type virus contamination. Pure recombinant clones were stored at -80°C for further investigation.

(B) Expression of Recombinant Iowa Strain Infectious Agent Proteins

Indirect immunofluorescence assay and radioimmunoprecipitation tests were used to evaluate expression.

Indirect immunofluorescence assay: Hi-five insect cells in a 24-well cell culture cluster plate were infected with wild-type baculovirus or recombinant baculovirus, or were mock-infected. After 72 hours, cells were fixed and stained with appropriate dilutions of swine anti-VR 2385 polyclonal antibodies, followed by fluorescein isothiocyanate-labelled (FITC-labelled) anti-swine IgG. Immunofluorescence was detected in cells infected with the recombinant viruses, but not in mock-infected cells or

cells inoculated with wild-type baculovirus. For example, Figure 12 shows HI-FIVE cells infected with the recombinant baculovirus containing the VR 2385 ORF-7 gene (Baculo.PRRSV.7), which exhibit a cytopathic effect.

5 Similar results were obtained with recombinant baculovirus containing ORF-5 (Baculo.PRRSV.5) and ORF-6 (Baculo.PRRSV.6; data not shown). Figures 13 and 14 show HI-FIVE cells infected with a recombinant baculovirus containing the VR 2385 ORF-6 gene and VR 2385 ORF-7 gene, respectively, stained with swine antisera to VR 2385, followed by fluorescein-conjugated anti-swine IgG, in which the insect cells are producing recombinant Iowa strain viral protein. Similar results were obtained with recombinant baculovirus containing ORF-5.

15 *Radioimmunoprecipitation:* Radioimmunoprecipitation was carried out with each recombinant virus (Baculo.PRRSV.5, Baculo.PRRSV.6 and Baculo.PRRSV.7) to further determine the antigenicity and authenticity of the recombinant proteins. HI-FIVE insect cells were mock-
20 infected, or alternatively, infected with each of the recombinant baculoviruses. Two days after infection, methionine-free medium was added. Each mixture was incubated for two hours, and then proteins labeled with ³⁵S-methionine (Amersham) were added, and the mixture was
25 incubated for four additional hours at 28°C. Radiolabeled cell lysates were prepared by three cycles of freezing and

thawing, and the cell lysates were incubated with preimmune or immune anti-VR 2385 antisera. The immune complexes were precipitated with Protein A agarose and analyzed on SDS-PAGE after boiling. X-ray film was exposed to the gels at -80°C, and developed. Bands of expected size were detected with ORF-6 (Figure 15) and ORF-7 (Figure 16) products.

EXPERIMENT III

Summary:

The genetic variation and possible evolution of porcine reproductive and respiratory syndrome virus (PRRSV) was determined by cloning and sequencing the putative membrane protein (M, ORF 6) and nucleocapsid (N, ORF 7) genes of six U.S. PRRSV isolates with differing virulence. The deduced amino acid sequences of the putative M and N proteins from each of these isolates were aligned with the corresponding sequences (to the extent known) of one other U.S. isolate, two European isolates, and other members of the proposed arterivirus group, including lactate dehydrogenase-elevating virus (LDV) and equine arteritis virus (EAV).

The putative M and N genes displayed 96-100% amino acid sequence identity among U.S. PRRSV isolates with differing virulence. However, their amino acid sequences varied extensively from those of European PRRSV isolates,

and displayed only 57-59% and 78-81% identity,
respectively. The U.S. PRRSV isolates were more closely
related to LDV than were the European PRRSV isolates. The
N protein of the U.S. isolates and European isolates shared
5 about 50% and 40% amino acid sequence identity with that of
LDV, respectively.

The phylogenetic dendrograms constructed on the basis
of the putative M and N genes of the proposed arterivirus
group were similar and indicated that both U.S. and
10 European PRRSV isolates were related to LDV and were
distantly related to EAV. The U.S. and European PRRSV
isolates fell into two distinct groups with slightly
different genetic distance relative to LDV. The results
suggest that U.S. and European PRRSV isolates represent two
15 different genotypes, and that they may have evolved from
LDV at different time periods and have existed separately
in U.S. and Europe before their association with PRRS was
recognized in swine.

ORF 6 encodes the membrane protein (M) of PRRSV, based
20 on the similar characteristics of the ORF 6 of EAV, ORF 2
of LDV, and the M protein of mouse hepatitis virus and
infectious bronchitis virus (Meulenberq et al, *Virology*,
192, 62-72 (1993); Conzelmann et al, *Virology*, 193, 329-339
(1993); Mardassi et al, *Abstr. Conf. Res. Workers in Animal*
25 *Diseases*, Chicago, IL, p. 43 (1993)). The product of ORF
7, the viral nucleocapsid protein (N), is extremely basic

and hydrophilic (Meulenberg et al, *Virology*, 192, 62-72
(1993); Conzelmann et al, *Virology*, 193, 329-339 (1993);
Murtaugh et al, *Proc. Allen D. Leman Swine Conference*,
Minneapolis, MN, pp. 43-45 (1993); Mardassi et al, *Abstr.*
5 *Conf. Res. Workers in Animal Diseases*, Chicago, IL, p. 43
(1993)).

The amino acid sequences encoded by ORF's 5, 6 and 7
of U.S. isolate VR 2385 and of the European isolate
Lelystad virus (LV) have been compared, and the identity
10 (i.e., the percentage of amino acids in sequence which are
the same) between the two viruses is only 54%, 78% and 58%,
respectively. Thus, striking genetic differences exist
between the U.S. isolate VR 2385 and the European isolate
LV (see U.S. application Serial No. 08/131,625, filed
15 October 5, 1993).

However, the U.S. isolate VR 2385 is highly pathogenic
compared to European LV. Thus, PRRSV isolates in North
America and in Europe appear to be antigenically and
genetically heterogeneous, and different genotypes or
20 serotypes of PRRSV may exist.

To further determine the genetic variation among the
PRRSV isolates, the putative M and N genes of five
additional U.S. PRRSV isolates with differing virulence
were cloned and sequenced. Phylogenetic trees based on the
25 putative M and N genes of seven U.S. PRRSV isolates, two
European PRRSV isolates and other members of the proposed

arterivirus group, including LDV and EAV, have been constructed.

PRRSV isolates (ISU-12 (VR 2385/VR 2386), ISU-22 (VR 2429), ISU-55 (VR 2430), ISU-79, ISU-1894 and ISU-3927 (VR 2431), each of which is disclosed and described in U.S. application Serial No. 08/131,625, filed October 5, 1993) were isolated from pig lungs obtained from different farms in Iowa during PRRS outbreaks, according to the procedure described in U.S. application Serial No. 08/131,625. A continuous cell line, ATCC CRL 11171, was used to isolate and propagate these viruses. All viruses were biologically cloned by three cycles of plaque purification prior to polynucleic acid sequencing.

Pathogenicity studies in caesarean-derived colostrum-deprived (CDCD) pigs, described in U.S. application Serial No. 08/131,625, showed that VR 2385, VR 2429 and ISU-79 were highly pathogenic, whereas VR 2430, ISU-1894 and VR 2431 were not as pathogenic. For example, VR 2385, VR 2429 and ISU-79 produced from 50 to 80% consolidation of the lung tissues in experimentally-infected five-week-old CDCD pigs necropsied at 10 days post inoculation, whereas VR 2430, ISU-1894 and VR 2431 produced only 10 to 25% consolidation of lung tissues in the same experiment.

Experimental Section:

Monolayers of ATCC CRL 11171 cells were infected with each of the PRRSV isolates at the seventh passage at an m.o.i. of 0.1. Total cellular RNA was isolated from infected cells by the guanidine isothiocyanate method (Sambrook et al, "Molecular Cloning: A Laboratory Manual," 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989)). The quality of RNA from each isolate was determined by Northern blot hybridization (data not shown) with a cDNA probe generated from the extreme 3'-end of the VR 2385 genome by the polymerase chain reaction (PCR) with primers PP284 and PP285 (SEQ ID NOS: 1 AND 2), as described in U.S. Application Serial No. 08/131,625. cDNA was synthesized from total cellular RNA with random primers using reverse transcriptase. The synthesized cDNA was amplified by polymerase chain reaction (PCR) as described previously (Meng et al, *J. Vet. Diagn. Invest.*, 5, 254-258 (1993)). Primers for RT-PCR were designed on the basis of a sequence in the genome of VR 2385 which resulted in amplification of the entire protein coding regions of the putative M and N genes (5' primer: 5'-GGGGATCCAGAGTTTCAGCGG-3' (SEQ ID NO:30); 3' primer: 5'-GGGAATTCACCGCATTC-3' (SEQ ID NO:33)). Unique restriction sites (EcoR I and BamH I) at the termini of the PCR products were introduced by conventional methods. A PCR product with the expected size of about 900 bp was obtained

from each of the virus isolates. Southern blot hybridization was then used to confirm the specificity of the amplified products.

The ³²P-labelled cDNA probe from VR 2385 hybridized with the RT-PCR products from each of the above virus isolates. The PCR products of the putative M and N genes from each of the PRRSV isolates were purified and cloned into vector pSK+ (Meng et al, *J. Vet. Diagn. Invest.* 5, 254-258 (1993)). Plasmids containing the full length putative M and N genes were sequenced with an automated DNA Sequencer (obtained from Applied Biosystems, Inc., Foster City California). Three to four cDNA clones from each virus isolate were sequenced with universal and reverse primers, as well as other virus specific sequencing primers (PP288: 5'-GCGGTCTGGATTGACGAC-3' (SEQ ID NO:5) and PP289: 5'-GACTGCTAGGGCTTCTGC-3' (SEQ ID NO:6), each of which is described in application Serial No. 08/131,625, and DP966: 5'-AATGGGGCTTCTCCGG-3' (SEQ ID NO:34)). The sequences were combined and analyzed by the MACVECTOR (International Biotechnologies, Inc.) and GENEWORKS (IntelliGenetics, Inc.) computer programs.

Analysis of the nucleotide sequences encoding the putative M and N proteins of the 5 U.S. PRRSV isolates indicated that, like LV (Meulenberq et al, *Virology*, 192, 62-72 (1993)) and VR 2385, the putative M and N genes of each of the five additional U.S. isolates overlapped by 8

base pairs (bp). Figure 17 shows the nucleotide sequence of ORF's 6 and 7 of six U.S. PRRSV isolates and of LV, in which the ISU-12 (VR 2385 and VR 2386) nucleotide sequence (SEQ ID NO:35) is shown first, and in subsequent sequences (SEQ ID NOS:36-41), only those nucleotides which are different are indicated. Start codons are underlined and indicated by (+1>), stop codons are indicated by asterisks (*), are indicated by (-), and the two larger deletions in the putative N gene are further indicated by (^).

Figures 18(A)-(B) show the alignment of amino acid sequences of the putative M (Fig. 18(A)) and N (Fig. 18(B)) genes of the proposed arterivirus group, performed with a GENEWORKS program (IntelliGenetics, Inc.), using the following parameters (default values): cost to open a gap is 5, cost to lengthen a gap is 25, minimum diagonal length is 4, and maximum diagonal offset is 10. The EAV M gene sequence was omitted because the relatively low sequence identity with PRRSV and LDV requires gaps in the alignments. The VR 2385/VR 2386 sequences (SEQ ID NOS:17 and 19) are shown first, and in subsequent sequences (SEQ ID NOS:43, 45, 47, 49, 51, 24, 53, 55, 57, 59, 61 and 26, respectively), only the differences are indicated. Deletions are indicated by (-), and the two larger deletions in the putative N gene are further indicated by (^).

Numerous substitutions in the nucleotide sequence were distributed randomly throughout the M and N genes in each of the five isolates, as compared to VR 2385. Most of the substitutions are third base silent mutations when converted to amino acid sequences (see Fig. 18). Insertions and deletions are found in the nucleotide sequences of the putative M and N genes when comparing the U.S. isolates to LV, but not found among the U.S. isolates (Fig. 17). For example, there are two larger deletions, 15 and 10 nucleotides each, in the putative N gene of the U.S. isolates as compared to the LV N genome (Fig. 17).

The deduced amino acid sequences of the putative M and N genes from the six Iowa strain PRRSV isolates are aligned with the corresponding N sequence of another U.S. isolate, VR 2332 (Murtaugh et al, *Proc. Allen D. Leman Swine Conference*, Minneapolis, MN, pp. 43-45 (1993)); two European PRRSV isolates, LV (Meulenberg et al, *Virology* 192, 62-72 (1993)) and PRRSV isolate 10 (PRRSV-10) (Conzelmann et al, *Virology*, 193, 329-339 (1993)); two LDV strains, LDV-C (Godney et al, *Virology*, 177, 768-771 (1990)) and LDV-P (Kuo et al, *Virus Res.*, 23, 55-72 (1992)); and EAV (Den Boon et al, *J. Virol.*, 65, 2910-2920 (1991)) (Fig. 18).

The amino acid sequences of the putative N gene are highly conserved among the seven U.S. PRRSV isolates (Fig. 18(B)), and displayed 96-100% amino acid sequence identity

(Table 1). However, the putative N proteins of the U.S. PRRSV isolates shared only 57-59% amino acid sequence identity with those of the two European isolates (Table 1), suggesting that the U.S. and the European isolates may represent two different genotypes.

The putative M protein of each of the U.S. isolates was also highly conserved, and displayed higher sequence similarity with the M proteins of the two European isolates (Fig. 18(A)), ranging from 78 to 81% amino acid identity (see Table 2 below). The putative N gene of each of the U.S. PRRSV isolates shared 49-50% amino acid sequence identity with that of the LDV strains, whereas the two European PRRSV isolates shared only 40-41% amino acid identity with that of the LDV strains (Table 2).

Two regions of amino acid sequence deletions, "KKSTAPM" (SEQ ID NO:62) and "ASQG" (SEQ ID NO:63), were found in the putative N proteins of each of the seven U.S. PRRSV isolates, as well as the two LDV strains and EAV, when compared to the two European PRRSV isolates (Fig. 18(B)). These results indicated that the U.S. PRRSV isolates are more closely related to LDV than are the European PRRSV isolates, and that PRRSV may have undergone divergent evolution in the U.S. and in Europe before their association with PRRS was recognized in swine (Murtaugh, *Proc. Allen D. Lemay Swine Conference, Minneapolis, MN*, pp. 43-45 (1993)).

The European isolates may have diverged from LDV for a longer time than the U.S. isolates, and hence may have evolved first. However, the amino acid sequence identity of the putative M gene between U.S. PRRSV isolates and LDV strains was similar to that between the European PRRSV isolates and LDV strains (Table 2). The putative M and N genes of the U.S. and European isolates of PRRSV shared only 15-17% and 22-24% amino acid sequence identity with those of EAV, respectively.

The sequence homology of PRRSV with LDV and EAV suggests that these viruses are closely related and may have evolved from a common ancestor (Plagemann et al, supra; Murtaugh, supra). The high sequence conservation between LDV and PRRSV supported the hypothesis that PRRSV may have evolved from LDV and was rapidly adapted to a new host species (Murtaugh, supra). Asymptomatic LDV infection were found in all strains of mice (Murtaugh, supra; Kuo et al, supra). However, many pig farms are infested with wild rodents (Hooper et al, *J. Vet. Diagn. Invest.*, 6, 13-15 (1994)), so it is possible that PRRSV evolved from LDV-infected mice, and was rapidly adapted to a new host, swine.

The evolutionary relationships of PRRSV with other members of the proposed arterivirus group were determined on the basis of the amino acid sequence of the putative M and N genes. Figure 19 shows a phylogenetic tree of the

Table 2. Pairwise comparison of the amino acid sequences among the putative nucleocapsid and membrane proteins of the proposed arterivirus group

VIRUS												
Virus	VR2385	ISU-22	ISU-55	ISU-79	ISU-1894	ISU-3927	VR2332	LV	PRRSV-10	LDV-P	LDV-C	EAV
VR2385	***	98	96	98	98	96	96	57	57	49	49	22
ISU-22	99	***	98	100	100	98	98	57	57	49	49	23
ISU-55	99	100	***	98	98	97	96	59	59	49	49	23
ISU-79	98	99	99	***	100	98	98	57	57	49	49	23
ISU-1894	99	100	100	99	***	98	98	57	57	49	49	23
ISU-3927	96	97	97	97	97	***	96	59	59	49	49	23
VR2332	N/A	N/A	N/A	N/A	N/A	N/A	***	57	57	50	49	22
LV	78	79	79	79	79	81	N/A	***	99	41	40	23
PRRSV-10	78	79	79	79	79	81	N/A	100	***	41	40	23
LDV-P	50	51	51	51	51	51	N/A	53	53	***	98	23
LDV-C	49	50	50	50	50	50	N/A	52	52	96	***	24
EAV	16	16	16	16	16	15	N/A	17	17	16	17	***

Note. *The values in the table are the percentage identity of amino acid sequences. N/A, not available.

^bNucleocapsid protein comparisons are presented in the upper right half and membrane protein comparisons are presented in the lower left half.

proposed arterivirus group based on the amino acid sequences of the putative M and N genes of this group. The phylogenetic tree for the N gene is essentially the same as that for the M gene. The length of the horizontal lines connecting one sequence to another is proportional to the estimated genetic distance between sequences, as indicated by the numbers given above each line. The UPGMA (unweighted pair group method with arithmetic mean) trees were constructed with a GENEWORKS program (IntelliGenetics, Inc.), which first clusters the two most similar sequences, then the average similarity of these two sequences is clustered with the next most similar sequences or subalignments, and the clustering continued in this manner until all sequences/isolates are located in the tree; both trees are unrooted.

The PRRSV isolates fall into two distinct groups. All the U.S. PRRSV isolates thus far sequenced are closely related and form one group. The two European PRRSV isolates are closely related and form another group. Both the U.S. and European PRRSV isolates are related to LDV strains and are distantly related to EAV (Fig. 19).

The evolution patterns for the putative N and M genes also suggest that PRRSV may be a variant of LDV. For example, the genetic distance of the U.S. PRRSV isolates is slightly closer to LDV than the European PRRSV isolates (Fig. 19), again suggesting that the U.S. and European

PRRSV may have evolved from LDV at different time periods and existed separately before their association with PRRS was recognized in swine. European PRRSV may have evolved earlier than U.S. PRRSV. It is also possible that the U.S. and European PRRSV could have evolved separately from different LDV variants which existed separately in the U.S. and Europe.

A striking feature of RNA viruses is their rapid evolution, resulting in extensive sequence variation (Koonin et al, *Critical Rev. Biochem. Mol. Biol.*, 28, 375-430 (1993)). Direct evidence for recombination between different positive-strand RNA viruses has been obtained (Lai, *Microbiol. Rev.*, 56, 61-79 (1992)). Western equine encephalitis virus appears to be an evolutionally recent hybrid between Eastern equine encephalitis virus and another alphavirus closely related to Sindbis virus (Hahn et al, *Proc. Natl. Acad. Sci. USA*, 85, 5997-6001 (1988)). Accordingly, the emergence of PRRSV and its close relatedness to LDV and EAV is not surprising. Although the capsid or nucleocapsid protein has been used for construction of evolutionary trees of many positive-strand RNA viruses, proteins with conserved sequence motifs such as RNA-dependent RNA polymerase, RNA replicase, etc., are typically more suitable for phylogenetic studies (Koonin et al, *supra*).

EXPERIMENT IV:

CLONING AND SEQUENCING OF cDNA CORRESPONDING TO ORF'S 2, 3
AND 4 OF PRRSV VR 2385.

5 The region including ORF's 2, 3, and 4 of the genome
of the porcine reproductive and respiratory syndrome virus
(PRRSV) isolate VR 2385 was cloned and analyzed. To clone
the cDNA of PRRSV VR 2385, ATCC CRL 11171 cells were
infected with the virus at a m.o.i. of 0.1, and total
cellular RNA was isolated using an RNA Isolation Kit
10 (Stratagene). The mRNA fraction was purified through a
Poly(A) Quick column (Stratagene), and the purified mRNA
was used to generate a cDNA library. A cDNA oligo dT
library was constructed in Uni-ZAP XR λ vector using a ZAP-
cDNA synthesis kit (Stratagene), according to the
15 supplier's instructions. Recombinant clones were isolated
after screening of the library with an ORF 4 - specific
hybridization probe (a 240 b.p. PCR product specific for
the 3' end of ORF 4; SEQ ID NO:64). Recombinant pSK +
contained PRRSV-specific cDNA was excised *in vivo* from
20 positive λ plaques according to the manufacturer's
instructions.

Several recombinant plasmids with nested set of cDNA
inserts with sizes ranging from 2.3 to 3.9 kb were
sequenced from the 5' ends of the cloned fragments. The
25 nucleotide sequence of SEQ ID NO:65 was determined on at
least two independent cDNA clones and was 1800 nucleotides

in length (Fig. 21). Computer analysis of the nucleotide and the deduced amino acid sequences was performed using GENEWORKS (IntelliGenetics, Inc.) and MACVECTOR (International Biotechnologies, Inc.) programs.

5 Three partially overlapping ORF's (ORF 2, ORF 3 and ORF 4) were identified in this region. ORF's 2, 3 and 4 comprised nucleotides 12-779 (SEQ ID NO:66), 635-1396 (SEQ ID NO:68) and 1180-1713 (SEQ ID NO:70), respectively, in the sequenced cDNA fragment.

10 A comparison of DNA sequences of ORF's 2, 3 and 4 of PRRSV VR 2385 with corresponding ORF's of LV virus (SEQ ID NOS:72, 74 and 76, respectively) is presented in Fig. 22. The level of nucleotide sequence identity (homology) was 65% for ORF 2, 64% for ORF 3 and 66% for ORF 4.

15 The predicted amino acid sequences encoded by ORF's 2-4 of PRRSV VR 2385 (SEQ ID NOS:67, 69 and 71, respectively) and of LV (SEQ ID NOS:73, 75 and 77, respectively) are shown in Fig 23. A comparison of PRRSV VR 2385 and LV shows a homology level of 58% for the protein encoded by
20 ORF 2, 55% for the protein encoded by ORF 3 and 66% for the protein encoded by ORF 4 (see Fig. 23).

EXPERIMENT V

An immunoperoxidase method of detecting PRRSV

25 Four 3-week-old colostrum-deprived PRRSV negative animals were inoculated intranasally with $10^{5.8}$ TCID₅₀ of

PRRSV U.S. isolate ATCC VR 2386 propagated on ATCC CRL 11171 cells. These pigs were housed on elevated woven-wire decks and fed a commercial milk replacer. Two pigs were necropsied at 4 days post inoculation (DPI) and two at 8 DPI.

At the time of necropsy, the right and left lungs of each pig were separated and inflated via the primary bronchus with 45 ml of one of four fixatives and then immersion fixed for 24 hours. The fixatives used in this experiment included 10% neutral buffered formalin, Bouin's solution, HISTOCHOICE (available from Ambresco, Solon, OH), and a mixture containing 4% formaldehyde and 1% glutaraldehyde (4F:1G). The tissues fixed in Bouin's were rinsed in five 30-minute changes of 70% ethyl alcohol after 4 hours fixation in Bouin's. All the tissues were routinely processed in an automated tissue processor beginning in 70% ethyl alcohol. Tissues were processed to paraffin blocks within 48 hours of the necropsy.

Sections of 3 micron thickness were mounted on poly-L-lysine coated glass slides, deparaffinized with two changes of xylene and rehydrated through graded alcohol baths to distilled water. Endogenous peroxidase was removed by three 10-minute changes of 3% hydrogen peroxide. This was followed by a wash-bottle rinse with 0.05 M TRIS buffer (pH 7.6) followed by a 5-minute TRIS bath. Protease digestion was performed on all tissue sections except those fixed in

HISTOCHOICE. Digestion was done in 0.05% protease
(Protease XIV, available from Sigma Chem., St. Louis, Mo.)
in TRIS buffer for 2 minutes at 37°C. Digestion was
followed by a TRIS-buffer wash-bottle rinse and then a 5-
5 minute cold TRIS buffer bath. Blocking for 20 minutes was
done with a 5% solution of normal goat serum (available
from Sigma Chem., St. Louis, Mo.).

The primary antibody used was the monoclonal antibody
SDOW-17 (obtained from Dr. David Benfield, South Dakota
10 State Univ.), diluted 1:1000 in TRIS/PBS (1 part TRIS:9
parts PBS (0.01 M, pH 7.2)). The monoclonal antibody SDOW-
17 recognizes a conserved epitope on the PRRSV nucleocapsid
protein (Nelson et al, *J. Clin. Microbiol.*, 31:3184-3189).
The tissue sections were flooded with primary antibody and
15 incubated at 4°C for 16 hours in a humidified chamber. The
primary antibody incubation was then followed by a wash-
bottle rinse with TRIS buffer, a 5-minute TRIS buffer bath,
and then a 5-minute TRIS buffer bath containing 1% normal
goat serum. The sections were flooded with biotinylated
20 goat anti-mouse antisera (obtained from Dako Corporation,
Carpintera, CA) for 30 minutes. The linking antibody
incubation was followed by three rinses in TRIS buffer, as
was done following primary antibody incubation. The
sections were then treated with peroxidase-conjugated
25 streptavidin, diluted 1:200 in TRIS/PBS, for 40 minutes,
followed by a TRIS buffer wash-bottle rinse and a 5-minute

TRIS buffer bath. The sections were then incubated with freshly-made 3,3'-diaminobenzidine tetrahydrochloride (DAB, obtained from Vector Laboratories Inc., Burlingame, CA) for 8-10 minutes at room temperature, and then rinsed in a distilled water bath for 5 minutes. Counterstaining was done in hematoxylin (available from Shandon, Inc., Pittsburgh, PA), and the sections were rinsed with Scott's Tap Water (10 g MgSO_4 and 2 g NaHCO_3 in 1 liter ultrapure water), then with distilled water. After dehydration, the sections were covered with mounting media, and then a coverslip was applied.

Two negative controls were included. Substitution of TRIS/PBS buffer in place of the primary antibody was done for one control. The other control was done by substituting uninfected, age-matched, gnotobiotic pig lungs for PRRSV-infected lungs.

Histological changes in infected tissues were characterized by moderate multifocal proliferative interstitial pneumonia with pronounced type 2 pneumocyte hypertrophy and hyperplasia, moderate infiltration of alveolar septa with mononuclear cells, and abundant accumulation of necrotic cell debris and mixed inflammatory cells in the alveolar spaces. No bronchial or bronchiolar epithelial damage was observed. However, there was necrotic cell debris in the smaller airway lumina.

Intense and specific staining in the cytoplasm of infected cells was observed in the formalin- and Bouin's-fixed tissues. Staining was less intense and specific in the 4F:1G-fixed tissues. There was poor staining, poor cellular detail, and moderate background staining in the HISTOCHOICE-fixed tissues. Background staining was negligible with the other fixatives. Cellular detail was superior in the formalin-fixed tissue sections and adequate in the Bouin's- and 4F:1G-fixed tissues.

The labeled antigen was primarily within the cytoplasm of sloughed cells and macrophages in the alveolar spaces (Fig. 24) and within cellular debris in terminal airway lumina (Fig. 25). When compared to sections from the same block stained with hematoxylin and eosin, it was determined that most of the labeled cells were macrophages, and some were likely sloughed pneumocytes. Lesser intensities of staining were observed in mononuclear cells within the alveolar septa and rarely in hypertrophied type 2 pneumocytes.

Using an immunoperoxidase technique on frozen sections, others were able to detect antigen in epithelial cells of bronchioles and alveolar ducts as well as within cells in the alveolar septa and alveolar spaces (Pol et al, "Pathological, ultrastructural, and immunohistochemical changes caused by Lelystad virus in experimentally induced infections of mystery swine disease (synonym: porcine

epidemic abortion and respiratory syndrome (PEARS)), " Vet. Q., 13:137-143). We were unable to detect antigen in brochiolar epithelium using the present immunoperoxidase method.

5 The present streptavidin-biotin complex (ABC) technique using a PRRSV monoclonal antibody can be modified as needed to identify PRRSV-infected porcine lungs. Both 10% neutral-buffered formalin and Bouin's solution are acceptable fixatives. Protease digestion enhances the
10 antigen detection without destroying cellular detail. This technique is therefore quite useful for the diagnosis of PRRSV-induced pneumonia of pigs, and for detection of PRRSV in lung tissue samples.

EXPERIMENT VI

15 An immunohistochemical identification of sites of replication of PRRSV

Summary: Four three-week-old caesarian-derived, colostrum-deprived (CDCD) pigs were inoculated intranasally with an isolate of porcine reproductive and respiratory syndrome
20 virus. All inoculated pigs exhibited moderate respiratory disease. Two pigs were necropsied at 4 days post inoculation (PI) and two at 9 days PI. Moderate consolidation of the lungs and severe enlargement of the lymph nodes were noted at necropsy. Moderate perivascular

lymphomacrophagic myocarditis was observed. Marked lymphoid follicular hyperplasia and necrosis was observed in the tonsil, spleen, and lymph nodes.

Porcine reproduction and respiratory syndrome virus antigen was detected by the present streptavidin-biotin immunoperoxidase method primarily within alveolar macrophages in the lung and in endothelial cells and macrophages in the heart. Macrophages and dendritic-like cells in the lymph nodes, spleen, tonsil, and thymus stained intensively positive for PRRSV nucleocapsid protein antigen as well.

Experimental section: Four pigs were snatched from the birth canal of a sow that was positive for PRRSV antibody by indirect immunofluorescent antibody (IFA) examination of serum. The pigs were taken to a different site, housed on elevated woven-wire decks and raised on commercial milk replacer. These pigs were bled at 0, 7, 14, and 21 days of age and found to be negative for PRRSV antibody by the IFA test. No PRRSV was isolated from the serum of the pigs or sow using MARC-145 cells (available from National Veterinary Services Laboratory, Ames, Iowa).

All four pigs were inoculated intranasally at 3 weeks of age with $10^{5.8}$ TCID₅₀ of PRRSV U.S. isolate ATCC VR 2385 propagated on ATCC CRL 11171 cells. Mild-to-moderate respiratory disease was observed from 3-9 days post

inoculation (DPI). Two pigs were necropsied at 4 DPI and two at 9 DPI. At 4 DPI, one pig evidenced 31% and the other 36% tan-colored consolidation of the lungs. At 9 DPI, the remaining two pigs evidenced 37% and 46% consolidation of the lungs, respectively. Lymph nodes were moderately enlarged and edematous.

Lymphoid tissues collected at necropsy included the tonsil, thymus, spleen, tracheobronchial, mediastinal, and medial iliac lymph nodes. Lymphoid tissues were fixed by immersion for 24 hours in 10% neutral buffered formalin, processed routinely in an automated tissue processor, embedded in paraffin, sectioned at 6 microns and stained with hematoxylin and eosin. Additional sections (including the lung tissue sections above) were cut at 3 microns and mounted on poly-L-lysine coated slides for immunohistochemistry.

The immunoperoxidase assay described in Experiment VI above was repeated. Briefly, after endogenous peroxidase was removed with 3% hydrogen peroxide, primary monoclonal antibody ascites fluid diluted 1:1000 in TRIS/PBS was added for 16 hours at 4°C in a humidified chamber. The monoclonal antibody SDOW-17 (obtained from Dr. David Benfield, South Dakota State Univ.), which recognizes a conserved epitope of the PRRSV nucleocapsid protein, was used. Biotinylated goat anti-mouse linking antibody (obtained from Dako Corporation, Carpinteria, CA) was added,

followed by treatment with peroxidase-conjugated streptavidin (obtained from Zymed Laboratories, South San Francisco, CA) and incubation with 3,3'-diaminobenzidine tetrahydrochloride (obtained from Vector Laboratories Inc., Burlingame, CA). The incubated sample was finally counterstained in hematoxylin.

Microscopic lesions included interstitial pneumonia, myocarditis, tonsillitis, and lymphadenopathy. One section of lung from each lobe was examined. The interstitial pneumonic lesions were characterized by septal infiltration with mononuclear cells, hyperplasia and hypertrophy of type 2 pneumocytes, and accumulation of macrophages and necrotic cell debris in alveolar spaces. These lesions were moderate and multifocal by 4 DPI and severe and diffuse by 9 DPI. Bronchi and bronchiolar epithelium was unaffected. PRRSV antigen was readily detected by immunohistochemistry in alveolar macrophages. Large dark-brown PRRSV antigen-positive macrophages were often found in groups of 5-10 cells. A few PRRSV antigen-positive mononuclear cells were observed within the alveolar septa. PRRSV antigen was not detected in any tissues of the negative control pigs.

One section of left and one section of right ventricle were examined. At 4 DPI, there were small, randomly distributed, perivascular foci of lymphocytes and macrophages. There was moderate multifocal perivascular lymphoplasmacytic and histiocytic inflammation by 9 DPI.

Moderate numbers of endothelial cells lining small capillaries of lymphatics throughout the myocardium stained strongly positive for PRRSV antigen (Fig. 26) at both 4 and 9 DPI. The PRRSV antigen-positive endothelial cells frequently were not surrounded by inflammatory cells at 4 DPI, but were in areas of inflammation at 9 DPI. A few macrophages between myocytes and in perivascular areolar tissue also stained strongly positive for PRRSV antigen.

A mild tonsillitis with necrosis was observed.

Necrotic foci of 1-10 cells with pyknosis and karyorrhexis were commonly observed in the center of prominent follicles and less often in the surrounding lymphoreticular tissue. Large numbers of lymphocytes and macrophages were observed within the crypt epithelium, and moderate amounts of necrotic cell debris were observed in crypts. PRRSV antigen was readily detected within cells in the center of hyperplastic follicles, in the surrounding lymphoreticular tissue, and within cells in the crypt epithelium (Fig. 27). Staining was also present amongst necrotic debris in the crypts. In all these sites, the PRRSV antigen-positive cells resembled macrophages or dendritic-like cells.

Thymic lesions were minimal. There were a few necrotic foci with pyknosis and karyorrhexis in the medulla. These foci tended to involve or be near thymic corpuscles. PRRSV antigen was frequently identified within

macrophages near these necrotic areas and less often within large isolated macrophages in the cortex.

Necrotic foci and single necrotic cells were evident with germinal centers of lymphoid nodules and in
5 periarteriolar lymphoid sheaths (PALS) of the spleen.

PRRSV antigen positive staining cells were concentrated in the center of lymphoid follicles and scattered throughout PALS. The positive cells generally had large oval nuclei and abundant cytoplasm with prominent cytoplasmic
10 projections, compatible with macrophages or dendritic cells. Lesser numbers of positive-staining fusiform-shaped cells in the marginal zone were observed. The size and location of these cells suggests that they are reticular cells.

15 The predominant lymph node changes were subcapsular edema, foci of necrosis in lymphoid follicles, and the presence of syncytial cells at the border of the central lymphoid tissue with the loose peripheral connective tissue. The high endothelial venules were unusually
20 prominent and often swollen. The syncytial cells had 2-10 nuclei with multiple prominent nucleoli and moderate eosinophilic cytoplasm. These cells did not appear to contain PRRSV antigen. Intense and specific cellular cytoplasmic staining was observed in the follicles. The
25 positive cells had large nuclei with abundant cytoplasm and prominent cytoplasmic processes (Fig. 27). These cells

resembled macrophages or dendritic cells. Lesser numbers of positive cells were observed in the perifollicular lymphoid tissue.

5 The lesion severity and the amount of antigen detected within various tissues was generally similar at 4 and 9 DPI. The gross size of the lymph nodes and the number of syncytial cells in lymph nodes were more prominent at 9 DPI than at 4 DPI. The amount of antigen detected in the heart was also greater at 9 DPI.

10 Tissues from age-matched uninfected CDCD pigs were used for histologic and immunohistochemical controls. Other negative controls for immunohistochemistry included using the same protocol less the primary PRRSV antibody on the infected pig tissues. PRRSV antigen was not detected
15 in any of the negative controls.

Conclusions: The immunohistochemical procedure described herein is useful for detecting PRRSV antigen in the lung, heart and lymphoid tissues of PRRSV-infected pigs. Severe interstitial pneumonia and moderate multifocal perivascular
20 lymphohistiocytic myocarditis was observed. Marked lymphoid follicular hyperplasia and necrosis of individual or small clusters of cells in the tonsil, spleen, and lymph nodes was also observed. PRRSV antigen was readily detected in alveolar macrophages in the lung and in
25 endothelial cells and macrophages in the heart.

Macrophages and dendritic-like cells in tonsil, lymph nodes, thymus, and spleen stained intensely positive for viral antigen as well.

PRRSV may replicate in the tonsil with subsequent viremia and further replication, primarily within macrophages in the respiratory and lymphoid systems of the pig.

EXPERIMENT VII

Diagnosing PRRS:

The present streptavidin-biotin immunoperoxidase test for detection of PRRSV antigen in tissues is quite useful to confirm the presence of active infection. 26 pigs were experimentally inoculated with ATCC VR 2385 PRRSV in accordance with the procedure in Experiments V/VI above. One section of each of the lungs, tonsils, mediastinal lymph nodes, and tracheobronchial lymph nodes from each pig was examined. The virus was detected by the immunoperoxidase assay of Experiment V in 23/26 lungs, 26/26 tonsils, 15/26 mediastinal lymph nodes, and 14/26 tracheobronchial lymph nodes.

The pigs in this experiment were killed over a 28 day period post-inoculation. The virus was detected in at least one tissue in every pig necropsied up to 10 days post inoculation.

A complete technique for the streptavidin-biotin based immunoperoxidase technique for PRRSV antigen detection in porcine tissues is described in Experiment V *infra*.

Briefly, after endogenous peroxidase removal by 3% hydrogen peroxide and digestion with 0.05% protease (Protease XIV, Sigma Chemical Company, St. Louis, MO), primary monoclonal antibody ascites fluid diluted 1:1000 in TRIS/PBS is added for 16 hours at 4°C in a humidified chamber. The monoclonal antibody used was SDOW-17 (Dr. David Benfield, South Dakota State Univ.), which recognizes a conserved epitope of the PRRSV nucleocapsid protein (Nelson et al, "Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies," *J. Clin. Micro.*, 31:3184-3189 (1993)).

Biotinylated goat anti-mouse linking antibody (Dako Corporation, Carpinteria, CA) is then contacted with the tissue, followed by treatment with peroxidase-conjugated streptavidin (Zymed Laboratories, South San Francisco, CA), incubation with 3,3'-diaminobenzidine tetrahydrochloride (Vector Laboratories Inc., Burlingame, CA), and finally staining with hematoxylin.

Particularly when combined with one or more additional analytical techniques such as histopathology, virus isolation and/or serology, the present tissue immunoperoxidase antigen detection assay offers a rapid and reliable diagnosis of PRRSV infection.

EXPERIMENT VIII

The pathogenicity of PRRSV isolates in 4-8 week old pigs was determined. The isolates were divided into two groups: (1) phenotypes with high virulence (hv) and (2) phenotypes with low virulence (lv) (see Table 3 below). For example, the mean percentage of lung consolidation of groups of pigs inoculated with a PRRSV isolate is shown in Table 4 below. The pathogenicity of a number of PRRSV isolates at 10 DPI is shown in Table 5 below. The results in Table 5 were statistically analyzed to verify the difference between hv and lv phenotypes, as determined by percentage lung consolidation.

Isolates characterized as high virulence produce severe clinical disease with high fever and dyspnea. In general, hv isolates produce severe pneumonia characterized by proliferative interstitial pneumonia with marked type II pneumocyte proliferation, syncytial cell formation, alveolar exudate accumulation, mild septal infiltration with mononuclear cells, encephalitis and myocarditis (designated PRRS-B hereinafter). Isolates characterized as low virulence do not produce significant clinical disease and produce mild pneumonia characterized predominately by interstitial pneumonia with septal infiltration by mononuclear cells, typical of classical PRRS (designated PRRS-A hereinafter).

Table 3: Characteristics and Pathogenicity of PRRSV Isolates

Virus Isolate	No. of Subgenomic mRNAs	mRNA 4	Severity of gross pneumonia* lesions	Microscopic Lesions**		
				Lesion Type in Lung	Heart	Brain
High Virulence (hv)						
VR 2385	6	Normal	++++	B	++++	++++
VR 2429	8	Normal	++++	B	++++	+++
ISU-28	ND	ND	+++	B	++++	++++
ISU-79	8	Normal	++++	B	+++	+++
ISU-984	ND	ND	+++	B	+++	+++
Low Virulence (lv)						
ISU-51	ND	ND	+	A	+	+
VR 2430	8	Normal	+	A/B	+	+
ISU-95	ND	ND	+	A	+	+
ISU-1894	6	Normal	+	A/B	+	+
VR 2431	6	Deletion	+	A/B	-	-
Lelystad***	6	Normal	+	A	+/-	+/-

*: (-) normal, (+) mild, (++) moderate, (+++) severe, (++++) very severe pneumonia.

**: PRRSV isolates produce two types of microscopic lung lesions: Type A lesions include interstitial pneumonia with mild septal infiltration with mononuclear cells typical of PRRS as described by Collins et al (1992); Type B lesions include proliferation of type II pneumocytes, and are typical of those described as PIP (Halbur et al 1993).

***: Pol et al, (*Vet. Quart.*, 13:137-143 (1991); Wensvoort et al, Antigenic comparison of Lelystad virus and swine infertility and respiratory syndrome virus. *J. Vet. Diagn. Invest.*, 4:134-138 (1992); Meulenber et al, Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. *Virology*, 192:62-72 (1993).

TABLE 4

VIRUS ISOLATE	Mean % Lung Consolidation Score at DPI*			
	3	10	21	28
VR-2385	29	77.3	37.3	6.0
VR-2386pp	20.5	77.5	25.0	0.0
ISU-22	26.5	64.8	36.5	11.0
ISU-984	7.25	76.0	21.0	0.5
ISU-3927	13.5	10.5	0	0.0
PSP-36	0	0	0	0.0
UNINOC	0	0	0	0.0

*: Score range is from 0-100% consolidation of the lung tissue.

TABLE 5

INOCULUM	NO. PIGS	Mean % Lung Consolidation at 10 DPI \pm S.D.
Uninfected	10	0 \pm 0
CRL 11171 Cell Line	10	0 \pm 0
ISU-51	10	16.7 \pm 9.0
ISU-55	10	20.8 \pm 15.1
ISU-1894	10	27.4 \pm 11.7
ISU-79	10	51.9 \pm 13.5
VR-2386pp	10	54.3 \pm 9.8
ISU-28	10	62.4 \pm 20.9

* Pathogenicity of PRRSV isolates ISU-28, VR 2386pp and ISU-79 were not significantly different ($p > 0.05$) from each other, but were different from that of ISU-51, ISU-55, and ISU-1894 ($p < 0.001$). All PRRSV isolates were significantly different ($p < 0.001$) from controls.

The precise mechanisms important in pathogenesis of PRRSV infection have not been fully delineated. However, alveolar macrophages and epithelial cells lining bronchioli and alveolar ducts have been shown to contain viral antigen by immunocytochemistry on frozen sections (Pol et al: Pathological, ultrastructural, and immunohistochemical changes caused by Lelystad virus in experimentally induced infections of mystery swine disease (synonym: porcine epidermic abortion and respiratory syndrome (PEARS)). *Veterinary Quarterly*, 13:137-143 (1991)).

The present immunocytochemistry test for the detection of PRRSV in formalin-fixed tissues (see Experiment VI *supra*) shows that PRRSV also replicates in alveolar epithelial cells and macrophages. The extent of virus replication and cell types infected by PRRSV isolates also appears to vary (see Experiment X below).

The role of different genes in virulence and replication is not precisely known. However, ORF's 4 and 5 appear to be important determinants of *in vivo* virulence and *in vitro* replication in PRRSV.

The results of cloning and sequencing ORF's 5, 6 and 7 of PRRSV isolate VR 2385 (see Experiment I *supra*) show that ORF 5 encodes a membrane protein (also see U.S. application Serial No. 08/131,625). A comparison of ORF's 5-7 of VR 2385 with ORF's 5-7 of Lelystad virus shows that ORF 5 is the least-conserved of the three proteins analyzed (see

Table 2 *supra*), thus indicating that ORF 5 may be important in determining virulence.

Based on Northern blot results, ORF 4 of lv isolate VR 2431 appears to have a deletion in mRNA 4 (also see Experiment V of U.S. application Serial No. 08/131,625).

EXPERIMENTS IX-XI

PRRSV (ATCC VR 2386) was propagated *in vitro* in ATCC CRL 11171 cells by the method disclosed in Experiment III of U.S. application Serial No. 08/131,625. The PRRSV isolate was biologically cloned by three rounds of plaque purification on CRL 11171 cells and characterized. The plaque-purified isolate (hereinafter "VR 2386pp", which is equivalent to VR 2386, deposited at the ATCC, Rockville Maryland, on October 29, 1992) replicated to about 10^6 - 10^7 TCID₅₀/ml at the 11th cell culture passage in CRL 11171 cells. Viral antigens were also detected in the cytoplasm of infected cells using convalescent PRRSV serum. VR 2386pp was shown to be antigenically related to VR 2332 by IFA using polyclonal and monoclonal antibodies to the nucleocapsid protein of VR 2332 (SDOW-17, obtained from Dr. David Benfield, South Dakota State University).

Several other virus isolates (VR 2429 (ISU-22), ISU-28, VR 2428 (ISU-51), VR 2430 (ISU-55), ISU-79, ISU-984, ISU-1894, and VR 2431 (ISU-3927)) were isolated and plaque-purified on CRL 11171 cell line. Virus replication in the

CRL 11171 cell line varied among PRRSV isolates (see Table 3 below). Isolate VR 2385 and plaque-purified isolates VR 2386pp, VR 2430 and ISU-79 replicated to 10^{6-7} TCID₅₀/ml, and thus, have a high replication (hr) phenotype. Other isolates, such as ISU-984, ISU-1894 replicated to a titer of 10^{4-5} TCID₅₀/ml, corresponding to a moderate replication (mr) phenotype. Isolates ISU-3927 and ISU-984 replicated very poorly on CRL 11171 cell line and usually yielded a titer of 10^3 TCID₅₀/ml, and thus have a low replication (lr) phenotype.

EXPERIMENT IX

The pathogenicity of several PRRSV isolates was compared in cesarean-derived colostrum-deprived (CDCD) pigs to determine if there was a correlation between *in vitro* replication and pathogenicity (also see Experiment V of application Serial No. 08/131,625. Four plaque-purified PRRSV isolates (VR 2386pp, VR 2429, ISU-984, and VR 2431), and one non-plaque-purified isolate (VR 2385) were used to inoculate pigs. An uninoculated group and an uninfected cell culture-inoculated group served as controls. Two pigs from each group were killed at 3, 7, 10, and 21 DPI. Three pigs were killed at 28 and 36 DPI. Biologically cloned PRRSV isolates VR 2386pp, VR 2429, and ISU-984 induced severe respiratory disease in the 5 week-old CDCD pigs, whereas VR 2431 did not produce any significant disease. Gross lung lesion scores peaked at 10 DPI (see Table 4) and

ranged from 10.5% consolidation (VR 2431) to 77% consolidation (VR 2385). Lesions were resolved by 36 DPI.

Microscopic lesions included interstitial pneumonia, encephalitis, and myocarditis (Table 3). The lv isolates also caused less severe myocarditis and encephalitis than the hv isolates.

In Figs. 28(A)-(C), photographs of lungs from pigs inoculated with (A) culture fluid from uninfected cell line CRL 11171, (B) culture fluids from cell line infected with lv isolate VR 2431, (C) or culture fluids from cell line infected with hv isolate VR 2386pp. The lung in Fig. 28(B) has very mild pneumonia, whereas lung in Fig. 28(C) has severe consolidation.

EXPERIMENT X

An additional experiment was conducted using a larger number of pigs to further examine the pathogenicity of PRRSV isolates and to obtain more statistically significant data. Results are shown in Table 5. Collectively, the results show that PRRSV isolates can be divided into two groups based on pneumopathogenicity. Isolates VR 2385, VR 2429, ISU-28, and ISU-79 have a high virulence (hv) phenotype and produce severe pneumonia. Isolates ISU-51, VR 2430, ISU-1894 and VR 2431 have a low virulence (lv) phenotype (Table 4) and produce low grade pneumonia.

PRRSV isolates also produce two types of microscopic lesions in lungs. The first type found generally in lv isolates is designated as PRRS-A, and is characterized by interstitial pneumonia with septal infiltration with mononuclear cells typical of PRRS (as described by Collins et al, Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J. Vet. Diagn. Invest.*, 4:117-126 (1992)). The second type of lesion, PRRS-B, is found in hv isolates and is characterized as proliferative interstitial pneumonia with marked type II pneumocyte proliferation, alveolar exudation and syncytial cell formation, as described in U.S. application Serial No. 08/131,625 and by Halbur et al, An overview of porcine viral respiratory disease. *Proc. Central Veterinary Conference*, pp. 50-59 (1993). Examples of PRRS-A and PRRS-B type lesions are shown in Figs. 28(A)-(C), in which Fig. 28(A) shows a normal lung, Fig. 28(B) are the lesions produced by PRRSV type A, and Fig. 28(C) shows the lesions produced by PRRSV type B.

The immunoperoxidase assay of Experiment V using monoclonal antibodies to PRRSV was used to detect viral antigens in alveolar epithelial cells and macrophages (see Fig. 29(A)). This test is now being routinely used at the Iowa State University Veterinary Diagnostic Laboratory to detect PRRSV antigen in tissues.

In Figures 29(A) - (B), immunohistochemical staining with anti-PRRSV monoclonal antibody of lung from a pig infected 9 days previously with VR 2385. A streptavidin-biotin complex (ABC) immunoperoxidase technique coupled with hematoxylin counterstaining were used. Positive staining within the cytoplasm of macrophages and sloughed cells in the alveolar spaces is clearly shown in Fig. 29(A), and within cellular debris in terminal airway lumina in Fig. 29(B).

EXPERIMENT XI

To determine if there was a correlation between biological phenotypes and genetic changes in PRRSV isolates, Northern blot analyses were performed on 6 PRRSV isolates.

Total intracellular RNA's from the VR 2386pp virus-infected CRL 11171 cells were isolated by the guanidine isothiocyanate method, separated on 1% glyoxal/DMSO agarose gel and blotted onto nylon membranes. A cDNA probe was generated by PCR with a set of primers flanking the extreme 3' terminal region of the viral genome. The probe contained 3' noncoding sequence and most of the ORF-7 sequence (see U.S. application Serial No. 08/131,625).

Northern blot hybridization revealed a nested set of 6 subgenomic mRNA species (Fig. 30). The size of VR 2386pp viral genomic RNA (14.7 kb) and the six subgenomic mRNA's,

mRNA 2 (3.3 kb), mRNA 3 (2.8 kb), mRNA 4 (2.3 kb), mRNA 5 (1.9 kb), mRNA 6 (1.4 kb) and mRNA 7 (0.9 kb), resembled those of LV, although there were slight differences in the estimated sizes of the genome and subgenomic mRNA's (Conzelmann et al, *Virology*, 193, 329-339 (1993), Meulenbergh et al, *Virology*, 192, 62-72 (1993). The mRNA 7 of the VR 2386pp was the most abundant subgenomic mRNA (see Fig. 30 and Experiment I above). The total numbers of subgenomic mRNA's and their relative sizes were also compared. The subgenomic mRNA's of three isolates had 6 subgenomic mRNA's, similar to that described for Lelystad virus. In contrast, three isolates had 8 subgenomic mRNA's (Fig. 30). The exact origin of the two additional species of mRNA's is not known, but they are located between subgenomic mRNA's 3 and 6 and were observed repeatedly in cultures infected at low MOI. Interestingly, an additional subgenomic mRNA has been detected in LDV isolates propagated in macrophage cultures (Kuo et al, 1992). We speculate that the additional mRNA's in cells infected with some PRRSV isolates are derived from gene 4 and 5 possibly transcribed from an alternate transcriptional start site. Additional studies are needed to determine the origin of these RNA's and their significance in pathogenesis of PRRSV infections.

Fig. 30 shows Northern blots of PRRSV isolates VR 2386pp (designated as "12"), VR 2429 (ISU-22, designated as

"22"), VR 2430, designated as "55"), ISU-79 (designated as "79"), ISU-1894 (designated as "1894"), and VR 2431, designated as "3927"). This data represents results from four separate Northern blot hybridization experiments. The VR 2386pp isolate (12) was run in one gel, ISU-1894 and VR 2431 were run in a second gel, VR 2430 and ISU-79 were run in a third gel, and ISU-22 was run in a fourth gel. Two additional mRNA's are evident in isolates VR 2429, VR 2430, and ISU-79.

The subgenomic mRNA 4 of VR 2431 (ISU-3927) migrates faster than that of other isolates in Northern blotting, suggesting a deletion. Interestingly, the isolate VR 2431 has lv and lr phenotypes and is the least virulent PRRSV isolate of the Iowa strains described herein. This suggests that gene 4 may be important in virulence and replication. As described above, genes 6 and 7 are less likely to play a role in expression of virulence and replication phenotypes.

In summary, PRRSV isolates vary in pathogenicity and the extent of replication in cell cultures. The number of subgenomic mRNA's and the amount of mRNA's also varies among U.S. PRRSV isolates. More significantly, one of the isolates, VR 2431, which replicates to low titer (lr phenotype) and which is the least virulent isolate (lv phenotype) among the Iowa strain PRRSV isolates described

herein, appears to have a faster migrating subgenomic mRNA 4, thus suggesting that a deletion exists in its ORF 4.

EXPERIMENT XII

COMPARISON OF THE PATHOGENICITY AND ANTIGEN DISTRIBUTION OF TWO U.S. PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS ISOLATES WITH THE LELYSTAD VIRUS

PRRSV-induced respiratory disease with secondary bacterial pneumonia, septicemia and enteritis are frequently observed in 2-10-week-old pigs (Halbur et al., "Viral contributions to the porcine respiratory disease complex," *Proc. Am. Assoc. Swine Pract.*, pp. 343-350 (1993); Zeman et al., *J. Vet. Diagn. Invest.* (1993)). Outbreaks may last from 1-4 months or become an ongoing problem on some farms where pig-flow through the unit is appropriate for shedding of the virus from older stock to younger susceptible animals that have lost passive antibody protection.

The severity and duration of outbreaks is quite variable. In fact, some herds are devastated by the high production losses (Polson et al., "Financial Impact of Porcine Epidemic Abortion and Respiratory Syndrome (PEARS)," *Proc. 12th Inter. Pig Vet. Soc.*, p. 132 (1992); Polson et al., "An evaluation of the financial impact of porcine reproductive and respiratory syndrome (PRRS) in nursery pigs," *Proc. 13th Inter. Pig Vet. Soc.*, p. 436

(1994)), while other herds have no apparent losses due to infection with PRRSV. This may be due to a number of possibilities, including virus strain differences, pig genetic susceptibility differences, environmental or housing differences, or production style (pig flow) of the unit.

This experiment compares the pathogenicity and antigen distribution of two U.S. strains (ISU-12 [VR 2385], ISU-3927 [VR 2431]) and a European strain (Lelystad virus, obtained from the National Veterinary Services Laboratory, P.O. Box 844, Ames, Iowa, 50010) in a common pig model to document similarities and differences that may explain the differences in severity of field outbreaks of PRRSV and help to better understand the pathogenesis of disease induced by PRRSV. (In the following experimental descriptions, "x/y" refers to the number of pigs "x" out of a particular group of pigs having "y" members.)

Materials and Methods

Experimental Design:

One hundred caesarian-derived-colostrum-deprived (CDCD) pigs of 4 weeks of age were randomly divided into 4 large groups of 25 pigs each and assigned to one of four isolated buildings. Within each building, the pigs were further divided into 3 separate rooms (11 pigs, 11 pigs, and 3 pigs per room). Each room within the buildings had

separate, automated ventilation systems. The pigs were housed on raised woven wire decks and fed a complete 18% protein corn and soybean meal based ration. Following challenge with a virus inoculum, the pigs were necropsied as detailed in Table 6 below at 1, 2, 3, 5, 7, 10, 15, 21 and 28 days post inoculation (DPI).

Virus Inocula Preparation:

Each virus was plaque-purified three times. Challenge doses were $10^{5.8}$ for VR 2385 and $10^{5.8}$ for VR 2431. The challenge dose of Lelystad virus was $10^{5.8}$.

Pigs were challenged intranasally by sitting them on their buttocks perpendicular to the floor and extending their neck fully back. The inocula was slowly dripped into both nostrils of the pigs, taking approximately 2-3 minutes per pig. Control pigs were given 5 mL of uninfected cell culture media in the same manner.

Clinical Evaluation

Rectal temperatures were taken and recorded daily from -2 DPI through 10 DPI. A clinical respiratory disease score was given to each pig daily from day 0 to 10 DPI, in accordance with the following 0-6 score range, similar to the respiratory distress analysis described above:

0 = normal

Table 6: Necropsy Schedule

Isolate	Room	1 DPI	2 DPI	3 DPI	5 DPI	7 DPI	10 DPI	15 DPI	21 DPI	28 DPI	Total
Lelystad	1	1	1	1	1	1	3	1	1	1	11
Lelystad	2	1	1	1	1	1	3	1	1	1	11
Lelystad	3						3				3
VR 2385	4	1	1	1	1	1	3	1	1	1	11
VR 2385	5	1	1	1	1	1	3	1	1	1	11
VR 2385	6						3				3
Control	7	1	1	1	1	1	3	1	1	1	11
Control	8	1	1	1	1	1	3	1	1	1	11
Control	9						3				3
VR 2431	10	1	1	1	1	1	3	1	1	1	11
VR 2431	11	1	1	1	1	1	3	1	1	1	11
VR 2431	12						3				3

- 1 = mild dyspnea and/or tachypnea when stressed
- 2 = mild dyspnea and/or tachypnea when not stressed
- 5 3 = moderate dyspnea and/or tachypnea when stressed
- 4 = moderate dyspnea and/or tachypnea when not stressed
- 5 = severe dyspnea and/or tachypnea when stressed
- 10 6 = severe dyspnea and/or tachypnea when not stressed

A pig was considered "stressed" by the pig handler after holding the pig under his/her arm and taking the pig's rectal temperature for approximately 30-60 seconds.

15 Other relevant clinical observations like coughing, diarrhea, inappetence or lethargy were noted separately, and are not reflected in the respiratory disease score.

Pathologic Examination:

Complete necropsies were performed on all pigs.

20 Macroscopic lung lesions were given a score to estimate the percent consolidation of the lung. Each lung lobe was assigned a number to reflect the approximate volume of entire lung represented by that lobe. Ten (10) possible points were assigned to each of the right anterior lobe,

25 right middle lobe, anterior part of the left anterior lobe, and caudal part of the left anterior lobe of the lung. The accessory lobe was assigned five (5) points. Twenty-seven

and one-half (27.5) points were assigned to each of the right and left caudal lobes to reach a total of 100 points. Gross lung lesion scores were estimated, and a score was given to reflect the amount of consolidation in each lobe. The total for all the lobes was an estimate of the percent consolidation of the entire lung for each pig.

Sections were taken from all lung lobes, nasal turbinates, cerebrum, thalamus, hypothalamus, pituitary gland, brain stem, choroid plexus, cerebellum, heart, pancreas, ileum, tonsil, mediastinal lymph node, middle iliac lymph node, mesenteric lymph node, thymus, liver, kidney, and adrenal gland for histopathologic examination. Tissues were fixed in 10% neutral-buffered formalin for 1-7 days and routinely processed to paraffin blocks in an automated tissue processor. Sections were cut at 6 μ m and stained with hematoxylin and eosin.

Immunohistochemistry:

Immunohistochemical staining was performed as described in Experiment VI above. Sections were cut at 3 μ m and mounted on poly-L-lysine coated slides. Endogenous peroxidase was removed by three 10-minute changes of 3% hydrogen peroxide. This was followed by a TRIS bath, and then digestion with 0.05% protease (Protease XIV, Sigma Chemical Company, St. Louis, Mo.) in TRIS buffer for 2 minutes at 37°C. After another TRIS buffer bath, blocking

was done for 20 minutes with a 5% solution of normal goat serum. Primary monoclonal antibody ascites fluid (SDOW-17, obtained from Dr. David Benfield, South Dakota State Univ.) diluted 1:1000 in TRIS/PBS was added for 16 hours at 4°C in a humidified chamber. After primary antibody incubation and a subsequent 5 minute TRIS bath containing 1% normal goat serum, the slides were flooded with biotinylated goat anti-mouse linking antibody (Dako Corporation, Carpintera, CA) for 30 minutes. The sections were washed with TRIS and treated with peroxidase-conjugated streptavidin (Zymed Laboratories, South San Francisco, CA) for 40 minutes, then incubated with 3,3'-diaminobenzidine tetrahydrochloride (Vector Laboratories Inc., Burlingame, CA.) for 8-10 minutes. Sections were then stained with hematoxylin.

Immunohistochemical controls substituted TBS for the primary antibody on all lung and lymphoid tissue sections. The same was done on other sections of other tissues interpreted as possibly positive. Uninfected control pigs also served as negative controls. No staining was detected in any of the control pig tissues. The amount of antigen was estimated according to the following scale: (0) = negative (no positive cells), (1) = isolated or rare positive staining cells (about 1-5 positive cells per histologic section), (2) = a relatively low number of positive cells, yet more abundant than isolated cells (for example, about 10-20 positive cells per histologic

section), (3) = a moderate number of positive cells (for example, about 40-80 positive cells per histologic section), and (4) = a relatively large number of positive cells (more than about 100 positive cells per histologic section).

Virus Isolation:

The same tissues from each of two pigs necropsied from each challenge group were pooled at 1, 2, 3, 5, 7, 14, 21, and 28 DPI. At 10 DPI, nine pigs were necropsied from each group, so three pools of the same tissues from three pigs were made from each challenge group. Serum was also similarly pooled.

Results

Clinical Disease:

The mean clinical respiratory disease score for each group is summarized in Table 7. Control pigs remained normal. Respiratory disease was minimal, and symptoms and histopathology were similar in the groups of pigs infected with Lelystad virus and VR 2431. By 2 DPI, a few pigs in each of these groups demonstrated mild dyspnea and tachypnea after being stressed by handling. From 5-10 DPI, more of the pigs in these groups demonstrated mild respiratory disease, and a couple pigs evidenced moderate, but transient, labored abdominal respiration. By 14 DPI,

Table 7: Mean Clinical Respiratory Disease Score

GROUP	0 DPI	1 DPI	2 DPI	3 DPI	4 DPI	5 DPI	6 DPI	7 DPI	8 DPI	9 DPI	10 DPI
Control	0	0	0	0	0	0	0	0.1	0	0.1	0
Lelystad	0	0.2	0.1	0.2	0.5	0.6	0.8	1.0	0.9	0.3	0.3
VR 2431	0	0	0.3	0.2	0.4	0.6	0.3	1.3	0.7	0.5	0.5
VR 2385	0	0.4	1.5	1.8	2.2	3.2	3.4	3.5	3.3	3.4	3.0

all pigs in the Lelystad virus (LV) and VR 2431 groups had recovered. Other transient clinical disease noted in a few pigs in these groups included chemosis, reddened conjunctiva, ear drooping, and patchy cyanosis of skin when stressed by handling. Coughing was not observed.

By 2 DPI, the VR 2385-challenged group demonstrated mild respiratory disease without having been stressed. By 5 DPI, all of the pigs in this group demonstrated moderate respiratory disease characterized by labored abdominal respiration and dyspnea when stressed. Some of the pigs in this group received respiratory distress scores of 5 or 6 for a 2- to 5-day period, and the mean clinical respiratory disease score peaked at 3.5/6 at 7 DPI. Respiratory disease was characterized by severe tachypnea and labored abdominal respiration, but no coughing was observed. The VR 2385 pigs generally were moderately lethargic and anorexic from 4-10 DPI. Other transient clinical signs included chemosis, roughed hair coats, lethargy, and anorexia. It took up to 21 DPI for the majority of the pigs in this group to fully recover.

Gross Lesions

Table 8 summarizes the estimated percent consolidation of the lungs for pigs in each group. Lung lesions in the Lelystad group and VR 2431 group were similar in type and extent. Lesions were first observed at 5 DPI for both

groups, and peaked at 15 DPI for the Lelystad challenged group and at 7 DPI for VR 2431 challenged group.

Individual scores ranged from 0-31 percent consolidation for the Lelystad group and 0-27 percent for the VR 2431

5 group. The mean estimated percent consolidation of the lung for the nine pigs necropsied at 10 DPI was 6.8 percent for Lelystad virus challenged pigs and 9.7 percent for the VR 2431 challenged pigs. The lesions were predominately in the cranial, middle and accessory lobes and in the
10 ventromedial portion of the diaphragmatic lobes. The consolidation was characterized by multifocal, tan-mottled areas with irregular, indistinct borders.

Table 8: Estimated Percent Consolidation of Lungs (0-100%)

GROUP	1 DPI	2 DPI	3 DPI	5 DPI	7 DPI	10 DPI	15 DPI	21 DPI	28 DPI
Control	0	0	0	0	0	0	0	0	0
Lelystad	0	0	0	4.8	2.3	6.8	8.8	1.8	0
VR 2431	0	0	0	2.5	8.5	9.7	7.5	0	0
VR 2385	0	4.3	10.5	15.3	46.5	54.2	12.5	6.0	0

15 Gross lymphoid lesions were more common than lung
20 lesions with both VR 2431 and LV. Lymphadenopathy was consistently observed in the mediastinal and middle iliac lymph nodes. These lymph nodes were tan in color, and from 5-28 DPI, were enlarged to 2-10 times their normal size. There often was at least one 1-5 mm fluid-filled cyst in

each of these lymph nodes. No other gross lesions were observed in the LV or VR 2431 groups.

5 The VR 2385 group had considerably more severe lung consolidation. The distribution of lung consolidation was similar to pigs infected with VR 2431 and LV, but either the entire cranioventral lobes or large coalescing portions of the cranial, middle, accessory and ventromedial diaphragmatic lobes were consolidated. There was no pleuritis and no grossly visible pus in airways. Estimated
10 percent consolidation of the lung 7-10 DPI ranged from 28% to 71%. The estimated mean score of the nine pigs necropsied at 10 DPI was 54.2% consolidation.

Lymphoid lesions in the VR 2385 group were generally similar to those observed in the other groups.
15 Additionally, lymph nodes along the thoracic aorta and in the cervical region were often 2-5 times the normal size. Spleens were also slightly enlarged and meaty in texture.

Several pigs in the VR 2385 group had moderately enlarged and rounded hearts with 10-30 mL of clear fluid in
20 the pericardial space. Some of these pigs also had 50-200 mL of similar fluid in the abdominal cavity. There was no visible exudate or fibrin in the fluid.

Microscopic Lesions:

Heart: Control pigs necropsied up to 10 DPI had no
25 evidence of myocardial inflammation. Several pigs

throughout the study had randomly distributed discrete foci of hematopoietic cells in the endocardium and myocardium. These hematopoietic cells (i) were observed in clumps of 10-30 cells, (ii) ranged in size from 8-20 microns, and
5 (iii) had large round-oval, dark staining nuclei with dense, clumped chromatin, multiple small nucleoli and scant amphophilic cytoplasm. At 10 DPI, 2/9 control pigs had mild multifocal perivascular lymphohistiocytic myocarditis. This was also observed in 1/2 pigs necropsied at 15 and 21
10 DPI, respectively.

VR 2431 inoculated pigs also had evidence of myocardial extramedullary hematopoiesis, similar to the controls. Myocarditis was first observed at 7 DPI, and was seen in 16/18 pigs necropsied from 7-28 DPI. The
15 myocarditis was mild, multifocal, usually perivascular and peripurkinje, and lymphohistiocytic. Inflammation was consistently found in the endocardium, often around or involving purkinje fibers. Inflammation in the epicardium and myocardium was most consistently either around vessels
20 or randomly distributed between muscle fibers. Myocardial degeneration, necrosis, or fibrosis was not evident. Low numbers of eosinophils were observed in the perivascular infiltrates in a 4/9 pigs at 9 DPI.

In the LV inoculated pigs, mild multifocal
25 extramedullary hematopoiesis was evident in most pigs up to 7 DPI. Mild myocarditis was first observed at 2 DPI and

was inconsistent and mild in pigs posted from 3-10 DPI. The pigs necropsied at 15 and 21 DPI had moderate multifocal myocarditis. The myocarditis was much less severe by 28 DPI. In all, 13/17 LV inoculated pigs
5 necropsied from 7-28 DPI had lymphohistiocytic myocarditis, which was mild-moderate, perivascular, peripurkinje or random in distribution. Fewer numbers of plasma cells and eosinophils were found in areas of inflammation from 10-28 DPI.

Moderate multifocal lymphohistiocytic myocarditis was
10 observed beginning at 10 DPI in all of the VR 2385 inoculated pigs. Severe myocarditis was observed in 2/9 pigs killed at 10 DPI and in 1/2 pigs killed at each of 15, 21, and 28 DPI, respectively. The more severe cases were characterized by multifocal-to-diffuse, lymphoplasmacytic
15 and histiocytic infiltrates that were most intense in the perivascular, peripurkinje, and endocardial regions. Lesser numbers of eosinophils and unidentifiable pyknotic cells were also observed in association with the inflammation. Myocardial degeneration, necrosis and
20 fibrosis were not evident.

Lung: Very mild lung lesions were observed in 2/25 of the control pigs. One pig necropsied at 5 DPI had mild multifocal septal thickening with lymphocytes, macrophages, and neutrophils. At 10 DPI, one pig had mild
25 peribronchiolar and perivascular lymphohistiocytic cuffing

and a mild increased number of macrophages and neutrophils in the alveolar spaces.

In the VR 2431 inoculated pigs, microscopic lung lesions were first detected at 2 DPI and were present in 20/25 of the pigs. All pigs necropsied on or after 7 DPI had microscopic lung lesions. The lesions, when present, were multifocal, mild (12/25) to moderate (8/25), generally most severe at 10 DPI and nearly resolved at 28 DPI. The multifocal interstitial pneumonia was characterized by three primary changes: septal thickening with mononuclear cells, type 2 pneumocyte hypertrophy and hyperplasia, and accumulation of normal and necrotic macrophages in alveolar spaces. These changes were present throughout the 28-day period. Mild-to-moderate peribronchiolar and perivascular lymphohistiocytic cuffing was observed in most pigs examined at 10-15 DPI but had apparently resolved by 28 DPI. Lung lesions were seldom observed in sections taken from the caudal lung lobe.

The LV inoculated pigs had microscopic lung lesions very similar to those of VR 2431 in distribution, type, and severity. Microscopic lung lesions were observed in 21/25 of the LV pigs. Lesions were first observed at 2 DPI and persisted throughout the 28 day period. The most severe lesions were seen in a few of the pigs necropsied at 10 DPI and in most of those necropsied at 15 and 21 DPI. The interstitial pneumonia was characterized mainly by septal

thickening with mononuclear cells, peribronchiolar and perivascular lymphohistiocytic cuffing, and accumulation of macrophages and necrotic debris in alveolar spaces. Type 2 pneumocyte hyperplasia and hypertrophy was less consistent and less severe than that observed in the VR 2431 inoculated pigs. Lung lesions were seldom seen in sections taken from the caudal lung lobe.

Every pig that was inoculated with VR 2385 and necropsied on or after 5 DPI had moderate-to-severe interstitial pneumonia. Mild multifocal lesions were observed at 2 DPI. The lesions became moderate and multifocal by 5 DPI, severe and diffuse from 7-10 DPI, and still moderate but patchy at 21 and 28 DPI. The interstitial pneumonia at all stages was also characterized by three primary changes (septal thickening with mononuclear cells, type 2 pneumocyte hypertrophy and hyperplasia, and accumulation of normal and necrotic macrophages in alveolar spaces). Of these three changes, the pneumocyte hypertrophy was most prominent and characteristic of VR 2385 inoculation. Peribronchiolar and perivascular lymphomacrophagic cuffing was mild by 5 DPI, moderate by 10 DPI, and nearly resolved by 28 DPI.

Immunohistochemistry

Both adrenal glands were examined from all pigs. Adrenal gland lesions were not observed in any of the

control, VR 2431 or LV inoculated pigs. In the VR 2385 inoculated pigs, 9/25 pigs had mild multifocal lymphoplasmacytic and histiocytic adrenalitis. Inflammation was usually observed in the medulla. Pyknotic cells and karryhectic debris were also observed amongst the inflammatory cells. Lymphoplasmacytic vasculitis and neuritis were also observed in the adrenal artery and nerve, respectively, in 3/28 of the VR 2385 inoculated pigs.

Nasal turbinate lesions were similar in type but differed in severity and frequency in the 4 groups of pigs. A low number (5/25) of the control and LV (5/25) inoculated pigs had mild rhinitis, observed at 10-21 DPI. The rhinitis was characterized by patchy dysplasia of the epithelium, with loss of cilia and mild multifocal subepithelial lymphohistiocytic and suppurative inflammation, with slight edema and congestion.

More of the VR 2431 inoculated pigs (17/25) had rhinitis. Lesions were mild at 5 DPI but moderate by 10 DPI. Epithelial dysplasia with intercellular edema, a blebbed or "tombstone" appearance of swollen superficial epithelial cells becoming pyknotic and apparently sloughing into the nasal cavity, and complete or partial loss of cilia on large patches of epithelium were observed. There was moderate diffuse subepithelial edema, dilated and congested veins, and multifocal infiltrates of lymphocytes,

plasma cells, macrophages and neutrophils. The inflammation was most intense near the locations where the ducts of submucosal mucous glands extended to the surface. Leukocytic exocytosis, especially of neutrophils, were frequently observed in dysplastic surface epithelium and along mucous ducts. By 21 DPI, the lesions had become mild, and were resolved by 28 DPI.

Rhinitis was first observed at 5 DPI in the VR 2385 inoculated pigs. A total of 20/25 pigs, and all 17 pigs necropsied on or after 7 DPI, had rhinitis similar to that observed in the ISU-3927 group, except that the lesion persisted throughout the 28 day period.

Tables 9, 10, and 11 summarize and compare the number of different tissues in which PRRSV antigen was detected for each of the challenge groups. No antigen was detected in the control pigs. Table 12 summarizes the estimated amount of antigen in some of the tissues that were tested.

Virus isolation

Virus isolation from various tissues is summarized in Table 13, where "Lg" refers to lungs, "LN" refers to lymph nodes, "Ht" refers to the heart, "Ser" refers to serum, "Tons" refers to tonsils, "Spln" refers to the spleen, "SI" refers to small intestine, and "Brn" refers to the brain.

Table 9: Immunohistochemistry for VR 2385

Tissue	1 DPI	2 DPI	3 DPI	5 DPI	7 DPI	10 DPI	15 DPI	21 DPI	28 DPI	Total
Lung	0/2	1/2	2/2	2/2	2/2	9/9	2/2	2/2	2/2	22/25
TBLN	1/2	2/2	2/2	2/2	2/2	3/9	0/2	1/2	0/2	13/25
Med LN	0/2	2/2	2/2	2/2	2/2	4/9	0/2	0/2	2/2	14/25
Iliac LN	1/2	2/2	2/2	2/2	2/2	5/9	0/2	0/2	0/2	14/25
Tonsil	2/2	2/2	2/2	2/2	2/2	9/9	2/2	2/2	2/2	25/25
Thymus	0/2	1/2	2/2	2/2	2/2	2/9	0/2	0/2	0/2	9/25
Spleen	0/2	2/2	2/2	2/2	0/2	3/9	0/2	1/2	0/2	10/25
# pos	2/2	2/2	2/2	2/2	2/2	9/9	2/2	2/2	2/2	25/25

Table 10: Immunohistochemistry for VR 2431

Tissue	1 DPI	2 DPI	3 DPI	5 DPI	7 DPI	10 DPI	15 DPI	21 DPI	28 DPI	Total
Lung	1/2	1/2	0/2	1/2	0/2	7/9	2/2	0/2	2/2	14/25
TBLN	0/2	2/2	1/2	2/2	2/2	1/9	0/2	0/2	0/2	8/25
Med LN	0/2	2/2	2/2	2/2	1/2	1/9	0/2	0/2	2/2	10/25
Iliac LN	0/2	2/2	2/2	2/2	1/2	1/9	0/2	0/2	0/2	8/25
Tonsil	1/2	1/2	1/2	2/2	1/2	9/9	2/2	2/2	2/2	21/25
Thymus	0/2	0/2	2/2	1/2	1/2	0/9	0/2	2/2	0/2	6/25
Spleen	0/2	0/2	0/2	0/2	0/2	0/9	0/2	0/2	1/2	1/25
# pos	1/2	2/2	2/2	2/2	2/2	9/9	2/2	2/2	2/2	25/25

Table 11: Immunohistochemistry for Lelystad virus

Tissue	1 DPI	2 DPI	3 DPI	5 DPI	7 DPI	10 DPI	15 DPI	21 DPI	28 DPI	Total
Lung	0/2	1/2	1/2	1/2	1/2	5/9	2/2	2/2	1/2	14/25
TBLN	1/2	1/2	1/2	0/2	1/2	5/9	0/2	0/2	0/2	9/25
Med LN	1/2	1/2	2/2	1/2	1/2	2/9	0/2	1/2	1/2	10/25
Iliac LN	0/2	1/2	2/2	0/2	1/2	0/9	0/2	0/2	0/2	4/25
Tonsil	2/2	2/2	2/2	2/2	2/2	7/9	2/2	2/2	2/2	23/25
Thymus	0/2	0/2	0/2	2/2	0/2	0/9	0/2	0/2	0/2	2/25
Spleen	1/2	1/2	0/2	0/2	0/2	4/9	0/2	0/2	1/2	7/25
# pos	2/2	2/2	2/2	2/2	2/2	8/9	2/2	2/2	2/2	25/25

Serology

All pigs challenged with LV virus were negative prechallenge and remained <1:20 through 7 DPI. By 10 DPI, 6/9 of the pigs necropsied were seropositive with titers ranging from 1:20 to 1:1280. Only 2/10 pigs had titers >1:20 (both were 1:1280). By 15 DPI, all pigs were positive and 5/6 were >1:320. By 21 DPI, titers of 1:1280 or 1:5120 were most common. The VR 2431 antibody titers were similar to those levels seen with the LV virus. With VR 2385, however, 9/9 were positive by 10 DPI and 7/9 were \geq 1:320. No PRRSV serum antibody was detected in control pigs.

Discussion

This Experiment clearly demonstrates differences in pathogenicity between PRRSV isolates, differences in PRRSV antigen distribution, and differences in the amount of PRRSV antigen in selected tissues. The low virulence Iowa strain isolate VR 2431 and the low virulence Lelystad virus were similar in these criteria. The Iowa strain VR 2385 isolate was considerably more virulent, and PRRSV antigen was detected in more tissues and in greater amounts as compared to LV and VR 2431.

The pattern of antigen distribution over time (Table 12) suggests that when pigs are infected oronasally, initial and continual replication of the virus may be in

Table 12: Mean score for intensity/amount of PRRSV antigen detected by immunohistochemistry

DPI	VR 2385							VR 2431							Lelystad Virus					
	CrVn Lung	Mid Lung	TBLN	Med LN	llic LN	Tonsil		CrVn Lung	Mid Lung	TBLN	Med LN	llic LN	Tonsil		CrVn lung	Mid Lung	TBLN	Med LN	llic LN	Tonsil
1	0	0	1.5	0	0.5	1.0		0.5	0	0	0	0	0.5		0	0	0.5	0.5	0	1.0
2	0.5	1.0	2.0	1.5	2.0	1.5		0.5	0	2.0	1.0	2.5	0.5		0.5	0.5	1.0	1.0	1.0	1.0
3	2.0	2.5	3.0	3.0	3.0	3.0		0	0	1.0	1.5	2.5	0.5		0.5	0.5	1.0	1.5	2.0	1.0
5	2.0	2.0	3.0	3.0	2.5	3.0		0.5	1	2.0	2.0	2.0	1.0		1.0	0.5	0	0.5	0	1.0
7	2.5	1.5	1.0	1.5	2.0	1.0		0	2	1.0	1.0	0.5	0.5		1.0	0	1.0	0.5	0.5	1.0
10	2.0	1.6	0.5	0.6	0.7	1.2		1.1	0.9	0.1	0.1	0.1	1.1		0.3	0.4	0.6	0.2	0	0.8
15	1.0	0	0	0	0	1.0		2.0	0.5	0	0	0	1.0		0.5	0.5	0	0	0	1.0
21	2.0	0.5	0.5	0	0	2.5		0	0	0	0	0	1.0		1.0	0	0	0.5	0	1.5
28	1.0	0	0	1	0	1.5		1.3	0	0	1.3	0	2.0		0.5	0	0	0.5	0	1.0

Antigen amount was estimated and scored as follows: (0) = negative, (1) = isolated or rare positive staining cells, (2) = low number of positive cells, (3) moderate number of positive cells; and (4) = large number of positive cells.

CrVn = Cranioventral lung lobe; Mid = middle lung lobe; TBLN = tracheobronchial lymph node; Med LN = mediastinal lymph node.

the tonsil and upper respiratory tract lymphoid tissues, with subsequent viremia by 24 hours PI. A small amount of antigen is detected in the lung by 24 hours PI and peaks by 5-7 DPI, but persists there for up to 28 days. Antigen is present in lymphoid tissues generally from 2-21 DPI.

Antigen is detected primarily within the macrophages and dendritic-like cells in lung, lymph nodes, tonsil, thymus and spleen.

EXPERIMENT XIII

COMPARATIVE PATHOGENICITY OF NINE U.S. PRRSV ISOLATES IN A 5 WEEK OLD CDCD PIG MODEL

Part (A) of this experiment demonstrates a consistent model to study PRRSV-induced respiratory and systemic disease in piglets (e.g., about 5 weeks old) and to characterize gross and microscopic lesions associated with the course of PRRSV-induced disease. Part (B) of this experiment uses the model to statistically compare the virulence of PRRSV isolates from herds with differing disease severity, and to specifically determine if these differences may be due to virus virulence characteristics.

Materials and Methods

Source of PRRSV isolates:

Live pigs or fresh tissues were received from 61 herds over a 3-year period from 1991-1993. All cases were

submitted for etiologic diagnosis of respiratory disease in pigs from 1-16 weeks of age. Some of the herds had concurrent reproductive failure, and some did not. The nine selected herds differed in size, production style, age of diseased pigs, time since initial disease was observed, and severity of the current disease outbreak. The clinical information from the selected farms is summarized in Table 14.

Table 14: PRRSV Herd Profiles

Isolate	Herd Size	Production Style	Age of Disease	Type of Disease
VR 2385	180 sows	F-Fin/CF	ALL	severe PRRS
ISU-79	40 sows	F-Fin/AIAO	ALL	severe PRRS
ISU-28	150 sows	F-Fin/CF	ALL	severe PRRS
ISU-1894	600 sows	F-FRP/CF	3-8 weeks	severe resp.
VR 2428	900 sows	F-FRP/AIAO	3-8 weeks	severe resp.
VR 2429	100 sows	F-Fin/CF	1-8 weeks	moderate resp.
ISU-984	600 sows	F-FRP/AIAO	3-6 weeks	moderate resp.
VR 2430	150 sows	F-Fin/CF	3-6 weeks	mild resp.
VR 2431	60 sows	F-Fin/AIAO	1-4 weeks	mild resp.

F-Fin = Farrow-to-Finish
F-FRP = Farrow-to-Feeder Pig
CF = Continuous Flow
AIAO = All-in-All-out

Inocula preparation

PRRSV isolates were plaque purified 3 times in accordance with the procedure described in Experiment I, section (I) (A) above.

Experimental pigs:

Four-week-old caesarean-derived-colostrum-deprived (CDCD) pigs were initially fed a commercial 22% protein pig starter containing spray-dried plasma protein for 7 days, then were switched to a second stage 18% protein corn-soybean meal based ration for the duration of the experiment. Pigs were housed in 10 feet x 12 feet concrete-floored, individually power-ventilated rooms.

Part (A): CDCD pig model:

Ninety-eight 4-week-old CDCD pigs were randomly divided into 7 rooms of 14 pigs each. The rooms were randomly assigned one of seven treatments as shown in Table 15. The treatment consisted of intranasal inoculation of $10^{5.7}$ TCID₅₀ of a PRRSV isolate (selected from plaque-purified PRRSV isolates VR 2385, VR 2428 [ISU-22], VR 2431 or ISU-984, unplaque-purified isolate ISU-12 [VR 2386]), intranasal inoculation of uninfected cell culture and media, or no treatment. Two pigs from each group were necropsied at DPI 3, 7, 20 and 21, and 3 pigs were necropsied from each group at DPI 28 and 36. Rectal temperatures were recorded daily from DPI -2 through DPI +14. A clinical respiratory disease score was given from DPI -2 through DPI 14. Scores range from 0-6, in accordance with the respiratory distress scale recited in Experiment XII. A piglet was considered "stressed" by the pig handler when holding the pig under his/her arm and

taking the rectal temperature for approximately 30-60 seconds. Other relevant clinical observations (e.g., coughing, diarrhea, inappetence or lethargy) were noted separately as observed. Additional clinical observations had no impact on the clinical respiratory score. Weights were recorded an DPI 0, 7, 14, 21 and 28.

Table 15: Part (A) Experimental Design

Inoculum	3 DPI	7 DPI	10 DPI	21 DPI	28 DPI	36 DPI	Total Pigs
VR 2385	2	2	2	2	3	3	14
ISU-984	2	2	2	2	3	3	14
VR 2428	2	2	2	2	3	3	14
VR 2431	2	2	2	2	3	3	14
VR 2386	2	2	2	2	3	3	14
Uninoculated Control	2	2	2	2	3	3	14
PSP-36 Cell Culture	2	2	2	2	3	3	14

Part (B): Comparative Pathogenicity:

Results from Part (A) established that gross lung lesions were most severe at 10 DPI for 4 of 5 PRRSV isolates. Part (B) was designed to collect and compare data from a larger number of pigs necropsied at 10 DPI. In this experiment, 105 4-week-old crossbred CDCD pigs were randomly divided into seven rooms, each with 15 pigs. Each room was randomly assigned a treatment. Treatments

consisted of intranasal challenge with $10^{5.8}$ TCID₅₀ of one of six plaque-purified PRRSV isolates (VR 2429 [ISU-51], ISU-79, VR 2430 [ISU-55], ISU-1894, ISU-28 or VR 2385) or PSP-36 uninfected cell culture and media. Ten pigs from each group were necropsied at 10 DPI, and 5 pigs from each group were necropsied at 28 DPI. Rectal temperatures were recorded from -2 DPI to +10 DPI, and weights were recorded at 0, 10 and 28 DPI. Clinical respiratory disease scores and other clinical signs were recorded as in Part (A) above.

Serology:

Part (A): Pigs were bled at 0, 10 and 28 DPI. The presence of PRRSV serum antibody was detected by the immunofluorescent antibody technique (IFA) as described by Benfield et al (*J. Vet. Diagn. Invest.*, 4:127-133 (1992)).

Part (B): Pigs were bled at 0, 3, 10, 16 and 28 DPI and tested by the IFA procedure of Part (A) for the presence of PRRSV serum antibody.

Virus Isolation:

Virus isolation was attempted from lung homogenates of all pigs killed at 3, 7, 10, 21 and 28 DPI (Part (A)). Virus isolation was also attempted from lung and from serum of all pigs separately in two-pig pools using CRL 11171 (PSP 36) cells (Part (B)).

Gross Pathology:

Complete necropsies were performed on all pigs. All organ systems were examined. An estimated percent consolidation of the lung of each pig was calculated based on the scoring system described in Experiment XII above, in which each lung lobe was assigned a number to reflect the approximate volume of entire lung represented by that lobe. Other lesions were noted accordingly.

Microscopic Pathology:

Sections were taken from all lung lobes described above, as well as from nasal turbinates, cerebrum, thalamus, hypothalamus, pituitary gland, brain stem, choroid plexus, cerebellum, heart, pancreas, ileum, tonsil, mediastinal lymph node, middle iliac lymph node, mesenteric lymph node, thymus, liver, kidney, and adrenal gland for histopathologic examination. Tissues were fixed in 10% neutral buffered formalin for 1-7 days and routinely processed to paraffin blocks in an automated tissue processor. Sections were cut at 6 μ m and stained with hematoxylin and eosin. Lesions in several tissues were graded in accordance with the following scale: (-) = normal, (+) = mild, (++) = moderate, (+++) = severe, and (++++) = very severe (see Table 19).

Results

Clinical disease - Part (A), CDCD pig model:

VR 2385 challenged pigs demonstrated the most severe clinical respiratory disease, with scores above 2.5/6.0 on 7-9 DPI (Table 16). The onset of respiratory disease was noted on 3 DPI, and symptoms and lesions continued through 14 DPI. Respiratory disease was characterized by labored and accentuated abdominal respirations and tachypnea. There was no coughing. The pigs became lethargic by 3 DPI, were anorexic by 5 DPI, and did not return to full feed and activity until after 14 DPI. Eyelid edema was noted in two pigs on 6 and 7 DPI.

VR 2428-challenged pigs had a later onset of respiratory disease (5 DPI), but severe respiratory disease occurred more quickly and for a longer duration than in ISU-12-inoculated pigs. VR 2428 produced respiratory scores greater than 3.0/6.0 on 7-13 DPI. The pigs were off feed and lethargic at 6-14 DPI. No other clinical signs were noted.

ISU-984-challenged pigs produced moderate-to-severe respiratory disease with gradual onset starting at 4 DPI. The pigs were scored 2-2.5/6.0 for respiratory disease from 7-10 DPI, and greater than 3.0/6.0 with a few scores of 4-5/6.0 on 11-14 DPI. Other clinical signs included lethargy, eyelid edema, and blotchy-purple transient discoloration of skin.

VR 2431-challenged pigs produced mild respiratory disease. Disease onset occurred at 5 DPI with the most severe respiratory clinical disease scores between 2 and 2.5/6.0 in some pigs at 7-8 DPI. The pigs appeared considerably better by 10 DPI and were completely normal by 14 DPI. Lethargy and anorexia were observed on 7-8 DPI.

Mean rectal temperatures were greater than 104°F for all challenged groups by 7 DPI, and remained above 104°F until after 10 DPI. This coincided with the period of most severe clinical respiratory disease. The control pigs remained clinically normal throughout the experiment.

Clinical disease - Part (B), Comparative pathogenicity:

Clinical respiratory disease scores and rectal temperatures are summarized in Table 17. VR 2429 produced very mild respiratory disease and the pigs appeared near normal through 10 DPI. VR 2430 induced mild dyspnea and tachypnea from 4-10 DPI, as well as lethargy and anorexia from 4-6 DPI. At 5-8 DPI, ISU-1894 produced moderate respiratory disease of short duration, and the pigs were generally recovered by 10 DPI. ISU-1894-inoculated pigs were also transiently lethargic and anorexic from 4-7 DPI. ISU-79 induced severe respiratory disease with labored respirations of increased frequency, accompanied by lethargy and anorexia from 4 DPI to 15 DPI. ISU-12 induced moderate tachypnea and dyspnea of long duration (4-28 DPI).

These pigs were also moderately lethargic and mildly anorexic over that time period.

Pigs in three groups (ISU-12, ISU-79, ISU-28) frequently exhibited transient, blue-purple discoloration of the skin when stressed by handling. ISU-28 produced severe respiratory disease similar to ISU-79, but had a later onset (at 7 DPI) and only a 5-day duration. Controls remained normal through 10 DPI.

Gross lesions - Part (A), CDCD pig model:

Gross lung lesions were scored and estimated as percent lung consolidation. Results are summarized in Table 16. The degree of consolidation ranged from 7.3% (ISU-984) to 29% (VR 2386) at 3 DPI, 20% (VR 2431) to 56.3% (VR 2386) at 7 DPI, 10.5% (VR 2431) to 77.5% (VR 2385) at 10 DPI, 0% (VR 2431) to 37.3% at 21 DPI, and 0% (VR 2431, VR 2385) to 11% (VR 2428) at 28 DPI. No grossly detectable lesions remained in any group at 36 DPI. No gross lung lesions were observed at any time in the control group.

The affected lung lobes were primarily in the anterior, middle, accessory, and ventromedial portion of the caudal lobes. The consolidated areas were not well demarcated. These areas were multifocal within in each lobe and had irregular and indistinct borders, giving the affected lobes a tan-mottled appearance.

Table 16: Part (A) Mean Gross Lung Consolidation

Isolate	3 DPI		7 DPI		10 DPI		21 DPI		28 DPI	
	Clin. Score	Gross Lung	Clin. Score	Gross Lung	Clin. Score	Gross Lung	Clin. Score	Gross Lung	Clin. Score	Gross Lung
VR 2386	0.5	29	3.1	56.3	3.5	77.3	2.0	37.3	0.5	6.0
VR 2385	0.5	20.5	2.3	35.5	2.0	77.5	0.5	25.0	0	0.0
VR 2428	0	26.5	2.4	35.0	3.5	64.8	2.0	36.5	2.5	11.0
ISU-984	0.5	7.3	2.3	21.8	3.5	76.0	2.0	21.0	0	0.5
VR 2431	0	13.5	2.3	20.0	1.5	10.5	0	0	0	0.0
PSP-36	0	0	0	0	0	0	0	0	0	0.0
Uninoc.	0	0	0	0	0	0	0	0	0	0.0

Gross lesions - Part (B), Comparative pathogenicity:

Gross lung lesions were estimated by percent lung consolidation, and are shown in Table 18.

Microscopic lesions - Part (A), CDCD pig model:

5 Results are shown in Table 19. VR 2385, VR 2386, VR 2428 and ISU-984 all induced similar microscopic lung lesions. They produced moderate-severe interstitial pneumonia, characterized by: (i) type II pneumocyte proliferation, (ii) septal thickening with mononuclear
10 cells, and (iii) accumulation of mixed alveolar exudate. VR 2431 induced only mild interstitial pneumonia with

septal thickening by mononuclear cells. Myocarditis was observed only in the VR 2386 inoculated pigs.

Virus Isolation - Part (A), CDCD pig model:

PRRSV was recovered from the lungs of all 11 pigs inoculated with VR 2386, from 9 of 11 pigs inoculated with VR 2385, from 6 of 11 pigs inoculated with ISU-984, from 9 of 11 pigs inoculated with VR 2431, from 0 of 11 pigs inoculated with cell culture controls, and from 0 of 11 uninoculated control pigs up to 28 DPI.

Serology - Part (A), CDCD pig model:

All of the PRRSV inoculated pigs had detectable PRRSV antibody titer of ≥ 640 by 10 DPI. None of the control pigs had detectable PRRSV antibody. Most of the PRRSV-inoculated pigs had titers of ≥ 2560 by 28 DPI.

Serology - Part (B), Comparative pathogenicity:

All of the PRRSV-inoculated pigs had PRRSV antibody titers of ≥ 64 by 10 DPI. Control pigs did not have detectable PRRSV antibody.

Discussion

The 5-week-old CDCD pigs inoculated intranasally with $10^{5.8}$ TCID₅₀ of PRRSV provide an excellent model to study and compare PRRSV-induced respiratory and systemic disease.

Significant differences ($p < .05$) were observed in the pneumopathogenicity data reported in Table 18. Based on the results herein and in Experiment XI above, the isolates could be grouped into high and low virulence groups as follows:

high virulence: VR 2385, VR 2386, VR 2429 (ISU-22),
ISU-28, ISU-984, ISU-79
low virulence: VR 2431, VR 2428 (ISU-51), VR 2430,
ISU-1894, LV

A PRRSV isolate may be considered to be a "high virulence" phenotype if it results in one or more of the following:

- (a) a mean gross lung consolidation at 10 DPI of at least 30%, and preferably, at least 40%;
- (b) moderate-to-very severe type II pneumocyte hypertrophy and hyperplasia, moderate-to-very severe interstitial thickening, moderate-to-very severe alveolar exudate, and the presence of syncytia; or
- (c) a mean respiratory distress score of at least 2.0 at some point in time from 10-21 DPI.

Where an isolate does not meet any of the above criteria, it may be considered a "low virulence" phenotype.

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

Table 17: Part (B) Mean Respiratory Distress Scores and Mean Rectal Temperature (°F)

Isolate	Mean Respiratory Distress Score							Mean Rectal Temperature						
	3 DPI	5 DPI	7 DPI	10 DPI	15 DPI	21 DPI	28 DPI	3 DPI	5 DPI	7 DPI	10 DPI	15 DPI	21 DPI	28 DPI
PSP-36	0	0	0	0	0	0	0	102.7	102.6	103.3	103.7	103.1	103.5	103.8
VR 2429	0	0.1	0.7	0.2	0	0.2	0	102.6	103.7	104.2	103.2	104.5	103.6	104.2
VR 2430	0	1.1	0.8	1.5	0	0	0	102.8	103.7	104.1	103.8	103.5	104.6	104.1
ISU-1894	0	2.5	1.5	1.1	0.5	0	0	102.7	104.4	104.3	103.3	103.9	104.4	103.9
ISU-79	0	3.5	3.8	2.9	1.5	0.5	1.0	103.6	104.9	104.6	103.7	103.4	103.5	103.8
VR 2385	0.2	1.5	1.4	1.4	1.0	2.4	0.8	102.2	104.3	103.9	103.5	103.7	104.2	103.8
ISU-28	0	1.0	1.3	3.1	0	0	0	102.6	104.2	104.0	104.8	104.0	103.8	103.9

Table 18: Part (B), Mean Gross Lung Consolidation and Standard Deviation

Inocula	Number of Pigs	Mean gross lung score 10 DPI	SD
PSP-36	10	0.0	0.0
ISU-28	10	62.4	20.9
VR 2385	10	54.3	9.8
ISU-79	10	51.9	13.5
ISU-1894	10	27.4	11.7
VR 2430	10	20.8	15.1
VR 2429	10	16.7	9.0

Table 19: Experiment XIII, part (A), CDCD pig model: Microscopic Lesion Summary at 10 DPI

Lesion	VR 2386	VR 2385	VR 2428	ISU-984	VR 2431	PSP-36 control
Type II pneumocyte proliferation	++++	+++	+++	+++	+	-
Syncytia	++	++	++	++	-	-
Interstitial thickening	++++	+++	+++	+++	+	-
alveolar exudate	+++	+++	+++	+++	+	-
myocarditis	+	-	-	-	-	-
encephalitis	+	-	-	-	-	-